THE MICROBIOLOGICAL SAFETY AND QUALITY OF FOOD

VOLUME I

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The Microbiological Safety and Quality of Food

Volume I

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Foreword

Worldwide interest in food microbiology has never been greater. Concerns over food safety and quality, as well as food preservation and manufacture, have elevated the importance of food microbiology to an all time high. Consequently, the demand for current and comprehensive information and knowledge about the microbial aspects of food also has risen to new heights.

In response to these needs, the editors, Barbara Lund, Tony Baird-Parker, and Grahame Gould, all internationally recognized leaders in the field, have brought together more than 80 experts in the microbiology of food to address their specific specialities and lend their expertise to understanding the challenges of food safety and quality. This two-volume text serves as an outstanding reference for individuals responsible for the production, processing, distribution, sale, and serving of food; for the control of hazards and spoilage of food products; for teaching or studying the principles of food microbiology; and for assessing and regulating the safety and quality of foods. Its global approach fits well with today's needs.

The essentials of microbiological safety and quality of food have been brought together in this one book and presented in four major sections. Part I includes an introductory chapter, which presents an overview of safe and stable food production. The section then covers fundamental principles and practical information about food preservation. The factors influencing microbial survival and growth are related to processes and methods used to control microorganisms that may be hazardous or degradative or both. The most recent approaches to preservation including emerging nonthermal treatments and predictive modeling are included. Knowledge of the microbial ecology of foods represents a basic approach to the safety and quality of foods. This is addressed in Part II. Experts on the contamination, spoilage, and preservation of specific commodity groups representing all the major categories of food have compiled comprehensive overviews in each of these chapters.

Volume II begins with Part III which covers extensively foodborne pathogens and disease. This section is introduced by a review of the aspect of foodborne disease surveillance including its importance and the limitations and strategy of epidemiological investigations and reporting systems. Then a series of chapters on the major recognized foodborne pathogens, as well as the less well-recognized and unusual foodborne microbial illnesses, is presented. Long-term consequences as well as economic impacts are also important topics that are covered. The comprehensive nature of the chapters is evident in the content that includes the history, the characteristics and pathogenicity of the microorganisms and the disease, general epidemiology and incidence in food, the methods of isolation, and the factors that influence control. Part IV builds upon this extensive foundation and focuses on assurance of microbiological safety and quality. It presents chapters on the preventive approaches of food quality systems including HACCP, on hygienic design of installations, and on the various aspects of sampling and detection of microorganisms in food. The book concludes with a chapter covering the most recent thoughts on risk assessment and microbiological criteria for foods.

This compilation and advice will prove extraordinarily useful to all who are responsible for the microbiological safety and quality of foods. The use of this material, especially with its global approach and the experience of all the authors involved, should serve to enhance the safety and quality of the food offered to the public, wherever they may be.

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Preface

Substantial reductions in the incidence of foodborne disease and of food spoilage will only be achieved by applying our existing and developing knowledge and understanding of the biology, physiology, ecology, and pathogenicity of foodborne microorganisms and of traditional, new, and emerging food processing and preservation technologies. There is an urgent need to disseminate this knowledge to those concerned with the production and manufacture of food and to policy makers responsible for food legislation, and to ensure that appropriate intervention policies are promoted and implemented.

There is a wealth of relevant information, which is spread widely through an extensive literature on food science and technology, on medical aspects of foodborne disease, and on general microbiology. In this book we have brought together the key elements of this information that relate to the microbiological safety and quality of food, in chapters written by internationally renowned experts in their particular fields. Our hope is that the comprehensive coverage collected and reviewed critically here will contribute to the evolution of improvements in food safety and quality.

It is our aim that this book will be of value to practicing food microbiologists, persons responsible for development, production, and control in the food industry, and to medical and public health professionals, legislators, and lecturers and advanced students of food microbiology.

We wish to thank all the authors who have contributed chapters to this book for their co-operation, hard work, and patience, and the staff of Aspen Publishers for their enthusiastic help and collaboration.

Barbara M. Lund
Tony C. Baird-Parker
Grahame W. Gould

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I

Principles and Application of Food Preservation Techniques

1 The Production of Microbiologically Safe and Stable Foods

Tony C. Baird-Parker

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1.1 HISTORY AND BACKGROUND

Mankind has learned over many millennia which plant and animal species are edible and how the most useful of these could be cultivated, harvested, and stored to provide a supply of safe and nutritious food throughout the year. The establishment of such a food supply enabled man to progress from being a hunter and gatherer of food (pre-7000 to 8000 B.C.) to become a farmer, food producer, and local trader, and finally to become urbanized and to trade foods with neighboring and distant countries.

During urbanization, it was recognized that certain practices established for the preparation and storage of foodstuffs could lead to food-associated illnesses. Thus, many religious taboos and the early public health acts were concerned with the avoidance of illness from the consumption of foods.[12] For instance, over a thousand years ago, the Byzantine Emperor Leo VI (886–911 A.D.) banned the consumption of blood sausages in order to reduce the high incidence of sausage poisoning (subsequently categorized as botulism) among his people. The law he applied concerning the consumption of blood carried a most severe penalty if it was disobeyed. It stated: "A person found to have prepared blood as food, whether he sells it or buys it, shall have all his property confiscated, and, after having been severely scourged and disgracefully shaved, shall be exiled for life."[36] In England, the Statute of Pillory and Tumbrel (1266–1267) required that "If any butcher do sell contagious flesh or that hath died of the murrain" [any infectious disease of cattle] be punished. The customary punishment was to be placed in the stocks with the offending meat buried under them.[12]

Various authors and bodies have ranked the hazards associated with consumption of foods, and while the order of some vary depending on the region of the world and when the ranking was done, one of the main causes of human morbidity and mortality is microbiological contamination. The most quoted ranking of hazards associated with foodstuffs is by Wodicka[70] who lists the following in order of decreasing importance for Western communities: microbiological contaminants, nutritional deficiencies, environmental contaminants, natural toxicants, pesticide residues, and food additives.

1.1.1 Spoilage

Microbial spoilage of foods is caused by the growth and metabolic activities of various microorganisms. The wide range of microbial associations and metabolites that lead to the spoilage of foods is considered in detail in the food commodity chapters (Chapters 17–36) and some of the main metabolites are listed in Table 1-1.

Although it was not until the mid-19th century that microorganisms were finally shown to cause food spoilage by Louis Pasteur and others, thousands of years before this, man had developed means of preventing spoilage based on the use of empiricism to identify suitable methods. These methods included many of those still used today such as drying, freezing, smoking, curing, and fermenting. The use of these procedures added variety to the diet, and allowed early man to store and to transport otherwise perishable foods, and thus played an important role in establishing the international trading of foodstuffs. This is now more than a 300 billion dollar business worldwide.

Probably the first food preservation patent was granted in 1691 to two Englishmen (Thomas Porter and John White).[20] As far as is known this patent, which was based on the use of heat, was never widely applied. The honor of successfully developing the first commercial process to sterilize food using heat belongs to Appert.[5] In 1810 he was awarded a patent and the prize offered by the French government for the successful development of a process that provided Napoleon's armies with ready-prepared food that could be stored for long periods of time at ambient temperatures. This invention formed the basis of the modern canned food business, which developed during the second part of the 19th century. Canned foods were thus among the first widely available convenience foods. Further milestones in the development of preserved foods are listed in Exhibit 1-1; methods of preservation are considered in detail in Chapters 2–15.

Despite considerable advances made in the preservation of foodstuffs, it has been estimated that between 10% and 50% of raw agricultural products (especially grains and vegetables) are still lost annually as a result of spoilage.[44,68] Some of the major quality

Table 1-1 Major Reactions Leading to Quality Deterioration of Foods

Reaction	*Example*	*Consequences*
Physical Change	Movement of water	Drying—toughening of texture
		Hydration—softening of texture; sogginess
		"Lumping" and aggregation of particulate foods
Chemical Change	Oxidation	Oxidative rancidity
		Color change
	Maillard reactions	Brown discoloration
		Texture change
Enzymic Activity	Polyphenol oxidase	Enzymic browning
	Lipoxygenase	Rancidity
	Lipase	Lipolytic rancidity
	Protease	Texture change
		Gelation
		Flavor change
	Amylase	Texture change
Microbial Activity	Growth of spoilage organisms	Food spoilage[*]
	Growth of toxigenic organisms	Foodborne intoxication[†]
	Growth of infective organisms	Foodborne infection[‡]
	Growth of functional organisms	Flavor change[§]
		Texture change
		Preservation

[*] **Minor products,** eg, thiols, esters, amines, and peroxides causing off-odors, flavor defects, discoloration, etc.
Major products, eg, lactic acid, acetic acid, H_2 and CO_2, causing souring, blowing, etc.
Enzymes, eg, proteases, lipases, and amylases causing textural and flavor changes, etc.

[†] **Growth and formation of toxic metabolites in food,** eg, neurotoxins by *Clostridium botulinum*, enterotoxins by *Staphylococcus aureus*.

[‡] **Presence or multiplication of infective microorganisms in food,** eg, *E. coli* 0157:H7 or *Listeria monocytogenes*.

[§] **Addition of lactic acid bacteria,** eg, to make fermented milk, vegetable and meat products; addition of **yeasts,** eg, to make breads, beers, and wines.

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Exhibit 1-1 Milestones—Technologies for the Production of Safe and Stable Food

1810	Appert's canning patent published (France)
1864	Pasteur—"Pasteurization" of wine (France)
1870	Pasteurization of milk introduced (Denmark)
1874	Use of pressurized retorts for commercial canning (US)
1880s	Transportation of frozen meat from Australia to England
1890	Pasteurization of milk introduced (US)
1905	Chlorination of drinking water (UK)
1908	Sodium benzoate approved as a food preservative (US)
1920–22	Thermal process for canned foods based on the "botulinum cook" (US)
1924	"Holder" process introduced for pasteurizing milk, based on the destruction of *Mycobacterium tuberculosis* (US)
1928	Controlled atmosphere storage of apples to extend shelf life (Europe)
1929	Food irradiation patent published
1929	Marketing of quick-frozen foods (US)
1938	Controlled atmosphere storage of meat introduced (UK)
1947	Ice cream pasteurization regulations (UK)
1955	Sorbic acid approved as a food preservative (US)
1957	Concept of use of water activity as food preservation parameter published (Australia)
1963	Liquid egg (pasteurization) regulations (UK)
1977	WHO/FAO approve use of food irradiation up to 10 kGy
1988	Commercial use of ohmic sterilization of food (UK)
1991	Commercial use of high pressure for food pasteurization (Japan)
1997	WHO/FAO approve use of food irradiation up to 75 kGy

Source: Adapted from J.M. Jay, *Modern Food Microbiology,* 5th ed., p. 5, © 1998, Aspen Publishers, Inc.

losses and spoilage changes occurring in foods are physical, for example, caused by the movement of water within food structures; some are chemical, for example, oxidation; and some enzymatic, for example, lipases. However, by far the majority of losses results from the activities of microorganisms (Table 1-1). Quality losses occur throughout the food chain, their rate and type depending on the food, storage conditions, and formulation.[29]

1.1.2 Foodborne Illness

As noted above, the association between the consumption of food and illness was recognized by early civilizations. Thus, Hippocrates (c. 460 B.C.) concluded that there was a connection between human disease and food consumption,[36] and one of the earliest references to the dangers of consuming the flesh of animals that had died from natural causes is contained in the biblical command: "and ye shall be holy men unto me: neither shall ye eat any flesh torn of the beasts in the field; ye shall cast it to the dogs" (Exodus 22:31).[12] Also, the relationship between the consumption of bread made with rye grain contaminated with the sclerotia of the fungus *Claviceps purpurea* (ergots) and disease was recognized during the 10th century. However, it was not until the late 19th century that proof was obtained that foodborne illness was caused by infectious and toxigenic microorganisms and that some of the specific causative microorganisms were first recognized.

The number of different microorganisms recognized to cause foodborne illness has increased from around 5 identified by the beginning of the 20th century, to 20 by the early 1950s to more than 40 by the early 1990s.[9] New foodborne agents continue to be identified and in the last 5 years at least six new ones have been reported. Most such agents are likely to have caused illness in humans for many years but were not recognized because of the absence of suitable detection methods or because some change in practice resulted in their increased presence in the human food supply chain.

Some of the years in which evidence of the association of a microorganism with foodborne illness was first reported are listed in Exhibit 1-2.

Since the 1950s, and somewhat parallel with the development of intensive farming practices,[9] a large increase has occurred in the number of reported cases and outbreaks of illness caused by well-established foodborne pathogens, such as salmonellas and campylobacters. The increasing number of reports of foodborne illness comes mainly from Western countries and from those few other countries that collect such statistics. While some of this increase is the result of improved reporting of cases and better methods of detection, the majority reflects a real increase in the incidence of illness (see Chapter 37). Although in most Third World countries data are not collected on such a basis that an assessment of the amount of illness or the causes can be made, foodborne illness is probably second only to malnutrition as the cause of death among children in these countries; it is sec-

Exhibit 1-2 Milestones—Some Dates of First Reported Evidence of Foodborne or Waterborne Transmission of the Following Pathogenic Agents

1884	*Vibrio cholerae*
1888	*Salmonella*
1896	*Shigella*
1897	*Clostridium botulinum*
1914	*Staphylococcus aureus*
1939	*Yersinia enterocolitica*
1945	*Clostridium perfringens*
1949	*Listeria monocytogenes*
1950	*Bacillus cereus*
1951	*Vibrio parahaemolyticus*
1976	*Cryptosporidium*
1977	*Campylobacter jejuni*
1978	Small round structured viruses
1982	Verocytotoxigenic *Escherichia coli*
1986	Cyclospora
1997	Bovine spongiform encephalopathy agent

ond only to respiratory disease as the cause of illness in Western countries.[9]

Some estimates of foodborne illness cases occurring worldwide are of extreme concern. These include an estimate made in the early 1980s that in Africa, Asia (excluding China), and Latin America up to 5 million deaths and 1 billion cases of gastroenteritis occur every year among children under the age of five.[43] While it is impossible to be certain how much of this illness and death results from the direct consumption of food contaminated with pathogenic microorganisms, it seems likely that such food is the main source. A more recent estimate of deaths among infants and young children in Third World countries suggests that 13 million die every year as a result of the combined effects of diarrheal diseases and malnutrition.[56] Estimates of food-associated illness in Western countries are also of considerable concern, with estimates that up to one in three people in these countries suffer from acute gastroenteritis annually[8,58]; 1%–5% of cases of illness lead to serious and chronic sequelae including rheumatoid conditions, atherosclerosis, and nutritional and malabsorption problems.[7,51] The World Health Organization (WHO) estimates that the annual incidence of foodborne illness worldwide is in excess of 1 billion cases and is increasing. The socioeconomic cost of such illness is extremely high as discussed in detail in Chapter 56.

Reasons for the increasing incidence of reported foodborne illness cases, in addition to those resulting from improved reporting and detection methods, have been variously attributed to a combination of the following factors[32,66,69]:

- ***Changes in agricultural practices,*** such as intensive farming, where breeding and husbandry practices have led to the rapid spread of human and animal pathogens throughout the herds and flocks in many countries. The current examples of such spread are *Salmonella enteritidis* PT4 in poultry and *S. typhimurium* DT 104 in cattle.
- ***Vertical integration of animal production processes and associated practices,*** eg, recycling waste products from slaughter houses back into the animal food chain, via animal feed, resulting in the build-up of agents such as salmonellas in poultry flocks (Chapter 20) and bovine spongiform encephalopathy (BSE)-causing prions in cattle herds (Chapter 57).
- ***Lifestyle changes,*** such as more travel by persons to countries where standards of hygiene are lower than those in their home countries, leading to exposure to microorganisms to which they have no immunity.[60] Increased consumption of foods (particularly snack foods) outside the home may also lead to exposure to foodborne pathogens. Such foods are often prepared under unhygienic and unsuitable conditions by ill-trained staff, leading to the sale of unsafe foods.
- ***Demographic changes,*** leading to the increased susceptibility to infection of numerically larger sections of the population such as the very young, the elderly, persons under medical care, and the immunocompromised; it has been estimated that 20% to 25% of persons in Western communities belong to this category.[55,62]
- ***Malnutrition*** among Third World populations, leading to increased susceptibility to disease.[55,56] This situation is made worse by civil unrest in many parts of the world, resulting in large numbers of people forced to leave their homes, and then exposed to extreme conditions, leading to the rapid spread of foodborne and waterborne illnesses such as cholera and dysentery.
- ***Globalization of the world's food supply,*** leading to longer supply chains (more persons and food handling procedures involving potentially increasing risks). Increased world trade in foods and food raw materials is an emerging concern.[45,53,63] Such foods increasingly comprise fresh produce and other perishable foods transported by air, in contrast to the preserved foods and raw materials for further processing traditionally imported by sea. Examples of fresh pro-

duce that have recently caused illness are lettuce exported from Spain causing bacillary dysentery in Norway, Sweden, and the United Kingdom (UK),[46] and raspberries exported from Guatemala causing cyclosporiasis in the United States (US) and Canada.[34]

Other factors claimed to possibly or potentially increase the incidence of foodborne illness include the following: global warming[13]; less use of additives, such as nitrite, to preserve foods; and the increased marketing of mildly preserved, less processed foods, such as modified atmosphere packed chill-stored cooked meats and mildly heated in-pack heated foods prepared by the "Sous vide cuisine" process.

Because the types of foods involved in foodborne illness very closely reflect the types and sources of foods consumed by a population, their microbial ecology, and the methods of preparation for consumption, there are significant differences from country to country.[9] While there is much published information on the types of foods involved worldwide, most of these data have not been collected and collated in a form that can be used to estimate the prevalence of foodborne illness or the types of microorganisms involved. In most Western countries, unprocessed poultry and red meats are mainly reported to be involved in foodborne illness, with less than 5% of reported incidents caused by manufactured foods.[15,62] However, where the latter foods are involved in an incident, the number of cases of illness can be very high. For example, in 1994 there was an incident in the US involving *Salmonella enteritidis* contamination of a pasteurized ice cream mix that resulted in an estimated 224,000 persons becoming ill.[33] Examples of estimated financial costs of food poisoning incidents resulting from commercially produced foods in the UK are given in Exhibit 1-3. Further examples of costs and incidents are presented in Chapter 56.

1.2 SOURCES OF FOODBORNE PATHOGENIC MICROORGANISMS

The recognition of the sources of foodborne pathogenic microorganisms and the understanding of the factors that may lead to them causing illness are important when considering the means for their control. For the most part, such organisms are zoonotic, ie, of animal origin, and include such organisms as *Escherichia coli* O157:H7 and *Campylobacter jejuni*, or are widespread in the environment (geonotic) such as *Listeria monocytogenes* and *Bacillus cereus*. The illnesses they cause generally result from the contamination of raw agricultural produce, often combined with poor hygienic practices during preparation for consumption. Such practices include the following: undercooking, cross-contamination onto other foods, recontamination of the cooked product, and storage of preprepared foods at temperatures and for time periods that allow microbial growth to occur.[15,60]

Some foodborne pathogens, such as *Salmonella typhi* and the hepatitis A virus, are specifically of human origin and contaminate foodstuffs during primary production via sewage, for example, or during preparation for consumption as a result of poor personal hygiene by a food handler who is a carrier. Human carriers are also of concern in the contamination of foods with *Staphylococcus aureus*, *Shigella* spp. and Norwalk and Norwalk-like viruses (Chapters 46, 47, and 52).

Exhibit 1-3 Examples of Costs of Foodborne Illness in the UK Caused by Commercially Produced Foods

Year	*Illness*	*Food Involved*	*Cost Estimate**
1964	Typhoid	Canned corned beef†	£30 million
1978	Botulism	Canned salmon†	£4.0 million
1979	Staphylococcal food poisoning	Canned corned beef†	£2.0 million
1987	Salmonellosis	Infant dried milk	£3.0 million
1989	Salmonellosis	Salami sticks†	£5.0 million
1989	Botulism	Hazelnut yogurt	£6.5 million

*Approximate costs (inflated to 1997 costs) based on available information on costs of investigations, losses of earnings of victims, medical costs, and industry costs (recall, destruction of stock, loss of business).

†Imported product.

Tables 1-2, 1-3, and 1-4 list foodborne pathogens found on slaughtered animals, fish, and vegetable crops, respectively, and some of the basic hygiene measures used for their control.

1.3 CONTROL OF FOODBORNE PATHOGENIC MICROORGANISMS

Prevention of foodborne infections and disease is a multifactored process requiring establishment of control throughout the whole food supply chain. The first line of defense against contamination by microbial pathogens is to prevent the pathogens from contaminating food animals and crops at the primary production stage. The second line of defense is to avoid further contamination and growth during harvesting and post-harvest handling, and where possible, to introduce one or more steps, during their processing or their use in a manufactured food that will inhibit or kill the contaminating microorganisms or destroy any preformed microbial toxins.

Table 1-2 Control of Contamination from Food Animals

Organism of Concern	*Control Measures*
Principal source: intestinal tract	
Campylobacter spp *Clostridium perfringens** *Clostridium botulinum** *Escherichia coli* *Listeria monocytogenes** *Salmonella* spp* *Yersinia enterocolitica**† *Cysticercus bovis* *Taenia* spp *Trichinella spiralis*, etc	Animal husbandry (including control of feed) Transport and slaughter hygiene Process hygiene Cooking prior to eating
Principal source: tissues and milk	
Brucella spp *Mycobacterium bovis*§ *Corynebacterium ulcerans* *Coxiella burnetii* *Toxoplasma gondii* *Staphylococcus aureus*‡ *Streptococcus pyogenes*‡ *Streptococcus zooepidemicus*	Immunization Storage Veterinary inspection Processing Cooking prior to eating

*Also frequently an environmental contaminant.

†Also found in association with mouth tissues.

‡Most strains causing foodborne illness are of human origin.

§Bovine strain of *M. tuberculosis*.

Source: Data from A.C. Baird-Parker and W.E. Crabb, Frozen Products—Hygienic or Hazardous? *Journal of the Royal Society of Health*, Vol. 99, pp. 103–106, 113, © 1979, Royal Society of Health.

Table 1-3 Control of Contamination from Water, Fish, and Shellfish

Organism of Concern	*Control Measures*
Principal source: water, intestinal tract, or tissues of fish or shellfish	
Aeromonas hydrophila *Clostridium botulinum** *Vibrio cholerae* non-01 *Vibrio parahaemolyticus* *Vibrio vulnificus* *Vibrio fluvialis* *Anisakis marina* *Diphlobotrinum lactum* *Plesiomonas shigelloides* Cyanobacteria	Harvesting Storage Processing Preparation for consumption (disinfection of drinking water)
Principal source: human excreta	
Enteric bacteria *Vibrio cholerae* Norwalk and Norwalk-like viruses *Entamoeba histolytica* *Giardia duodenalis* *Cryptosporidium* species	Protect water sources from contamination Treat water to remove microbial contamination Protect shellfish growing areas Apply purification procedures

*Mostly nonproteolytic (Group II) strains.

Source: Data from A.C. Baird-Parker and W.E. Crabb, Frozen Products—Hygienic or Hazardous? *Journal of the Royal Society of Health*, Vol. 99, pp. 103–106, 113, © 1979, Royal Society of Health.

The first line of defense, while potentially the most effective, can be problematic because of the large number of possible sources of microbial contamination of food crops and animals prior to their harvest. This is best illustrated by the Salmonella Transmission Cycle shown in Figure 1-1, which shows the many potential sources of salmonella contamination of food animals.[10]

Treatments to make farm animals more resistant to infection by foodborne pathogens by blocking their attachment to the animal's intestinal tract are under evaluation by various research groups. Methods being studied include the use of competitive exclusion,[59] the development of vaccines,[54] and the long-term possibility of introducing resistance genes into an animal's gene pool.[9]

Where control of microbiological contamination cannot be achieved at the primary production stage, some control can be obtained during a manufacturing or processing step, or by the preparer or consumer in the case of a raw food cooked prior to con-

Table 1-4 Control of Contamination from Plant Crops

Organism of Concern	*Control Measures*
Principal source: soil and vegetation	
Bacillus cereus	Protect crops from damage during harvest and storage
Bacillus subtilis	
Clostridium botulinum	
Listeria monocytogenes	Control storage conditions
Mycotoxigenic molds	Process control
	Preparation for consumption
Principal source: human and animal excreta	
Enteric bacteria	Avoid irrigating or washing crops with contaminated water
Enteroviruses	
Hepatitis A virus	
Small-round-structured viruses	Avoid use of human and animal excreta as fertilizer
Helminths	Hygienic handling of salad crops
Protozoa	

Source: Data from A.C. Baird-Parker and W.E. Crabb, Frozen Products—Hygienic or Hazardous? *Journal of the Royal Society of Health,* Vol. 99, pp. 103–106, 113, © 1979, Royal Society of Health.

sumption. However, control achieved at later stages is seldom as effective as control at the primary production stage because contamination becomes more widely spread and more difficult to control. This leads to the potential contamination of food handling areas, of additional products, and of food handlers. An exception to this general rule is the pasteurization of milk, which is demonstrably a very effective procedure for destroying infective pathogens. When pasteurization is combined with hygienic handling pre- and post-pasteurization, excellent control of the microbiological hazards associated with milk and milk-based products results (Chapter 22). This introductory chapter will only consider the basic strategies for control. However, further procedures for controlling microorganisms in processed and manufactured foods are found in Chapters 2–16. Control requirements for the main food commodities are discussed in Chapters 17–36.

1.3.1 Strategies for Control

Whether a microbiologically contaminated food can be safely consumed depends on the numbers and types of pathogenic microorganisms or the amount(s) of microbial toxins present at the point of consumption and the susceptibility to infection or intoxication of the person consuming that food. The purpose of all control procedures is to ensure that potentially hazardous levels of microorganisms or toxins are not present in a food at the point of consumption. Similar control strategies apply to pre-

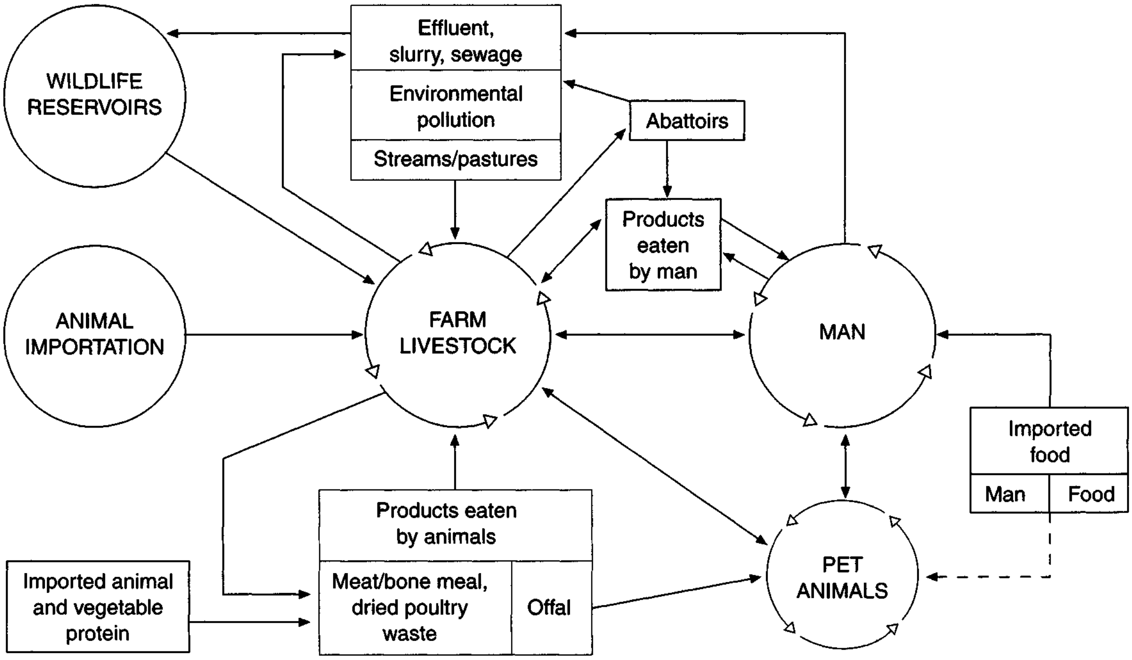


Figure 1-1 Cycle of Salmonella Transmission. *Source:* Reprinted with permission from A.C. Baird-Parker, Foodborne Salmonellosis, *The Lancet,* Vol. 336, pp. 1231–1234, © 1990, The Lancet Ltd.

venting or slowing the growth of spoilage microorganisms and thus preventing their metabolic products from causing spoilage.

Strategies for control of microbiological hazards in foodstuffs have evolved over many years, and this process of evolution continues with development of structured and quantitative risk assessment and hazard analysis techniques. These techniques are fully described and discussed in Chapters 58 and 65 and are summarized in Section 1.4 of this chapter.

The traditional approach to food control used by both food producers and the regulatory authorities has been mainly to inspect a production unit for compliance with General Hygienic Practice (GHP) requirements (often defined in law) and where appropriate to take samples of product to the laboratory for microbiological and other forms of testing.[39] Good Hygienic Practice requirements include basic requirements for the following: hygienic design, construction, operation, and sanitation of food production premises and equipment; the hygiene of operations used in the preparation, processing, storage, and use of raw materials and products; and the education and training of operatives in good personal hygiene and good hygienic practices[2,27] (Chapters 58 and 59). Good Hygienic Practice requirements are usually included in Good Manufacturing Practice (GMP) documents.

Traditionally, food producers have employed quality assurance or control personnel to check that GMP requirements are met and to take samples for microbiological and other forms of testing. The regulatory authorities have employed inspectors to monitor food producers and distributors, with the basic aim of enforcing food hygiene legislation and other regulations relating to the production and sale of foodstuffs. The usefulness of these procedures has been subject to considerable scrutiny in recent years. One of the main reasons for this is that despite the large amount of new hygiene legislation enacted in many countries in recent years, the number of cases of foodborne illness has continued to rise. Also, the increasing complexity and speed of modern food processing equipment (much of which is enclosed and cannot easily be inspected) makes traditional inspection procedures even less effective. Microbiological testing, the other linchpin of traditional control, has increasingly been demonstrated to provide poor assurance that production is under control. As the outcome of any testing is generally only known long after production has been completed and the product packed, the result can have no direct influence on the controls applied during production. Also for statistical reasons, the confidence of decisions as to product acceptability based on microbiological testing will always be poor (Chapter 60).

The costs involved in setting up and running modern food production plants and facilities are very high. Hygiene measures to ensure that foods produced are safe and wholesome are a significant element of these costs. Much of the existing food hygiene legislation is essentially GHP requirements and contains general phrases such as "all articles, fittings, and equipment with which food comes into contact shall be kept clean"; "where necessary, disinfected, sufficient for the purposes intended"; "where necessary appropriate facilities must be available" (European Economic Community [EEC] 93/43 Annex I). Such imprecise phrases, while necessary in legislation designed to meet the basic GHP/GMP requirements of large sectors of the food industry, require interpretation by inspectors checking for compliance and by plant owners wishing to set up food production facilities that comply with legislation. Their impreciseness has the potential to lead to much misunderstanding and wasted effort by inspectors and food plant owners and often result in the nonoptimal implementation of hygiene measures and poor cost benefit.

Government bodies such as the Codex Alimentarius Commission[17] have recognized that for many foods more precise advice on the hygienic design and control of food production plants is needed by both food producers and regulatory authorities. The Commission, therefore, recommends that in addition to meeting basic GHP requirements, specific Codes of Hygienic Practice should be developed by the Codex Alimentarius Food Hygiene Committee for the production of microbiologically sensitive foodstuffs, and that the principles of hazard analysis critical control point (HACCP) be applied throughout the food supply chain and to all foodstuffs.[27] Similar recommendations are contained in the European Union General Food Hygiene Directive[2] and are incorporated in the laws of many countries.

The proactive approach of HACCP, based on the application of structured hazard analysis and the identification of specific means of control of the identified microbiological hazards associated with the production of a specific foodstuff, contrasts sharply with the generally applied traditional approach to control described above. The application of HACCP is based on seven "principles."[27] These principles are as follows:

1. Conduct a hazard analysis to identify the microbiological and other product safety hazards of concern in a particular food operation.

2. Determine the Critical Control Points (CCPs) (places, procedures, etc.) where an identified hazard can be controlled.
3. Establish critical limits for control at the CCPs (that separate acceptable from unacceptable control).
4. Establish a system to monitor control of the CCP (to respond to a control deviation before a critical limit is exceeded).
5. Establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control.
6. Establish procedures for verification to confirm that the HACCP system is working effectively.
7. Establish documentation concerning all procedures and records appropriate to these principles and their application.

The application of these principles is fully described and illustrated in Chapter 58.

The wide adoption of the principles of HACCP by regulatory authorities and industry as an essential part of food control is based on recognition by all involved in the food chain of the power of this procedure and the advantages gained by its application. By traditional inspection, an authority will get little more than a snapshot of the effectiveness of the hygiene control procedures in place in a food plant at the time of inspection. In contrast, where a HACCP plan has been properly developed, implemented, verified, and documented, the authorities can inspect the plan to obtain an accurate picture of the control measures in place.[57] They also are able to understand the basis for these control measures and are able to review the records obtained of monitoring control at the Critical Control Points to ensure control within the defined Critical Limits. Inspectors can also see what actions were taken when these limits were exceeded. The advantages to industry applying HACCP are as follows:

- focusing technical resources into those operations and procedures that are critical to ensuring product safety
- providing a framework for managing safety
- providing better security of product safety and reduced risks of product failure and financial losses
- enabling product safety features to be designed into a product and its associated process at its design stage.

It is generally agreed that HACCP is the most cost-effective means of ensuring food safety and reducing product losses due to spoilage (Chapter 58).

The application of HACCP has resulted in less use of microbiological criteria as a primary means of control. However, microbiological criteria will continue to be used by regulatory authorities for border control (particularly in the absence of other information indicating that proper control has been applied during production and distribution of an imported foodstuff), and may also be used to audit production within a country. In addition, microbiological criteria will continue to be used by industry in purchasing agreements and for some internal control purposes. It is important that if microbiological criteria continue to be used as a means of food control that they should be properly formulated and their limitations fully understood by their proposers and users. The requirements for establishing and using microbiological criteria are fully described in Chapter 65.

1.4 ASSESSMENT OF MICROBIOLOGICAL RISKS

It is increasingly accepted by regulatory authorities and by industry that microbiological control requirements for foodstuffs should be firmly based on an understanding of the microbiological risks that need to be controlled. Risk assessment has been described as the science of understanding hazards, the likelihood that they will occur, and the consequences of their occurrence.[1] The assessment of microbiological risks associated with foodstuffs has been the concern of microbiologists throughout most of the 20th century. In the 1920s, such assessments were used in the United States to establish safe heat processes for canned foods, based on the destruction of *Clostridium botulinum* spores,[24] and for pasteurized milk based on the destruction of cells of *Mycobacterium tuberculosis,* and later, *Coxiella burnetii.*[48] More recently, safe heat processes have been established for *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Chapters 43 and 44). Microbiological criteria, based on a form of risk assessment, were introduced by the International Commission on the Microbiological Specifications for Foods (ICMSF) in the early 1970s and are used for establishing microbiological criteria by regulatory authorities and by the food industry throughout the world.[27,40]

The recently implemented World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement)[3] requires that countries signatory to the agreement base their laws concerned with protecting human, animal, and plant health on a process of risk assessment, with the aim of ensuring that health protection measures are scientifically based and are not used as disguised restrictions to trade. Thus, the SPS Agreement requires food safety legislation to be scientifically based and requires risk assessment to be applied, for example, when introducing microbiological requirements for controlling imported foods. This requirement has fueled renewed interest in developing methods for assessing microbiological risks on a quantitative basis.[16,49] The approaches to, and the difficulties in, applying quantitative risk assessment procedures to quantify microbiological risk are described and discussed in detail in Chapter 65. However, one of the main problems is the general absence of sound data on the relationship between a particular concentration of pathogenic microorganism and the probability of this concentration of microorganisms causing illness if it is consumed in a food, ie, dose-response data. In addition to the inherent virulence of individual strains, there are many factors affecting the infectivity and the illness-causing potential of a foodborne pathogen.[19] These are listed in Table 65-2 of Chapter 65 and illustrate the large range of different and interacting factors that can affect infectivity and disease.

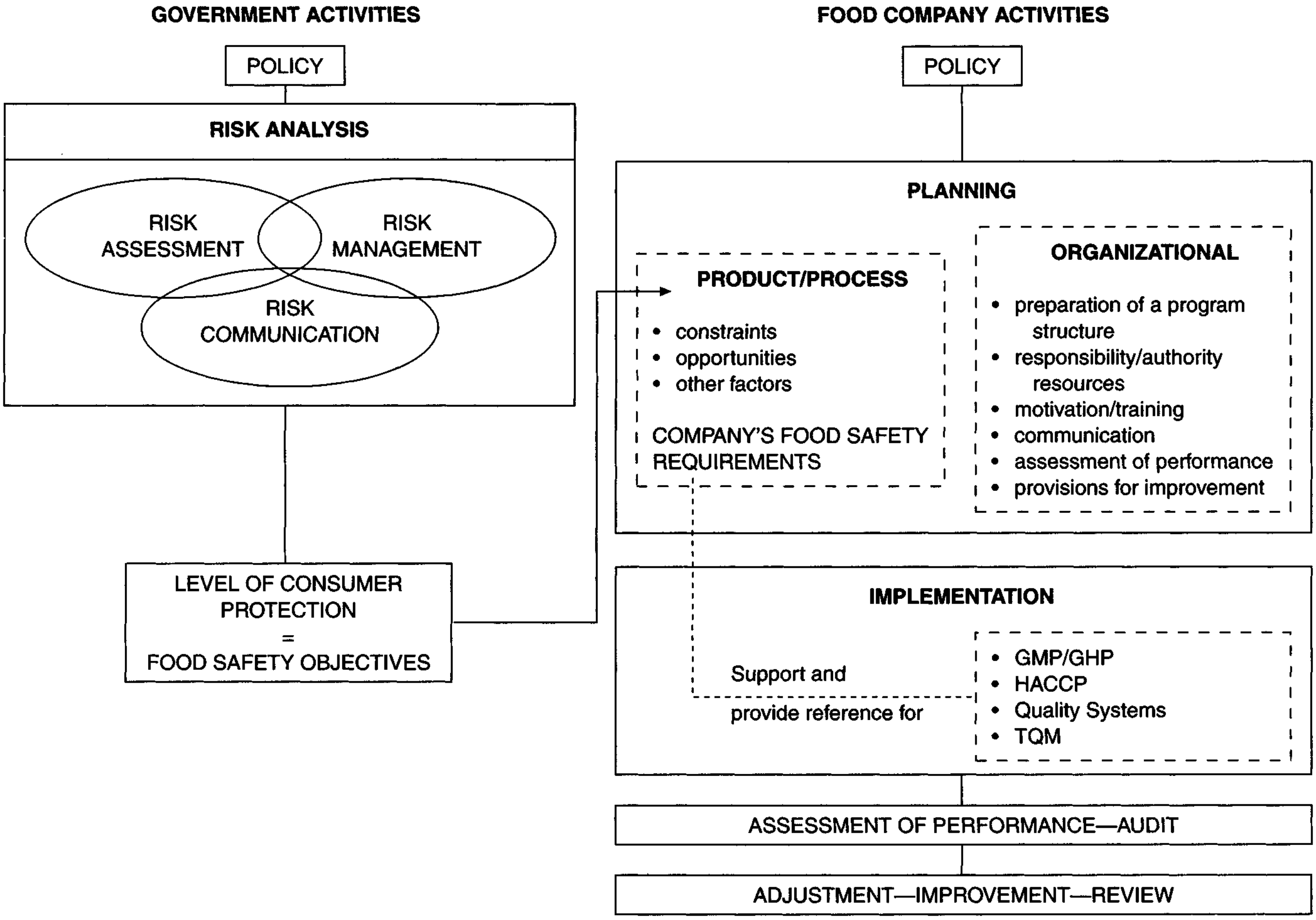
To collect reliable quantitative information on the effect of these factors on infectivity and the subsequent initiation of illness by a particular microbial pathogen is a daunting task. When considering a microbiological risk assessment for a particular food, the following knowledge should be required:

1. Types and numbers of foodborne pathogenic microorganisms, including toxic products of their metabolism, that may be present in a food raw material or ingredient, or that may be introduced during its transformation into a food product.
2. Effect of any processing (such as heating, curing, drying, acidulation, or addition of preservatives), or packaging eg, modified atmosphere packaging, on the survival or destruction of any pathogenic microorganisms or toxins as introduced in item #1 above.
3. Effect of conditions during storage and distribution such as freezing or chilling of the foodstuff and the effect of any procedures used for its preparation for consumption on the increase, decrease, or survival of any pathogenic microorganisms as introduced in item #1 or surviving (item #2).
4. Likely susceptibility of individuals in the consumer group for which the product is targeted, eg, infant, child, or hospital patient, to infection or intoxication by the microorganisms or toxins present in the foodstuff at the time of consumption.

To collect, collate, and assess the microbiological and medical knowledge for identification of the maximum concentration of an agent that is acceptable in a foodstuff requires input from several expert groups. Such groups could be industry- or government-led depending on the aim and scope of the particular type of assessment undertaken. For instance, a body responsible for assessing the microbiological risks associated with a particular pathogen or toxin in a foodstuff would be concerned with the collection of epidemiological, medical, microbiological, and toxicological information concerning the probability and extent of illness arising from the presence of an amount of the agent in foods consumed by a particular population. Such a risk assessment body would be independent, involving mainly academia and public health experts, with some input from industry on such matters as food consumption patterns and procedures generally applied to the processing or preparation of target foods for consumption. After establishing that a risk exists that needs managing, a risk management group would be set up to identify options for reducing any unacceptable risks to acceptable levels. This independent group would have greater government, consumer, and industry input so that policy and consumer issues can be considered and technically achievable options for control can be identified. Thus, the risk management group would be responsible for taking the output from the risk assessment body that has made a scientifically based estimate of the likelihood of a particular microbiological hazard causing harm, and converting this output, ie, the risk estimate, into a series of quantified control measures, eg, food safety objectives, that can be then be communicated to industry (part of the process of risk communication).

The processes of risk assessment, risk management, and risk communication are known collectively as risk analysis and the principles for performing these processes for microbiological and other biological risk assessments for foodstuffs have been subject to much recent international debate.[4,18,25,26] The relationship between governmental activities involved in risk analysis and the use of the output of such a risk analysis by a food producer to develop an internal control system is shown in Figure 1-2.

Microbiological risk assessment procedures are still in their infancy and are not sufficiently well developed to provide reliable and quantitative esti-



Note: GMP/GHP, good manufacturing practice/good hygienic practice; HACCP, hazard analysis critical control point; TQM, total quality management.

Figure 1-2 Interaction between Governmental Activities and a Company's Food Safety Activities. *Source:* Reprinted with permission from *Food Safety Management Tools,* © 1998, International Life Science Institute, European Branch, Brussels, Belgium.

mates of microbiological risk (Chapter 65). However, much progress has been made in applying microbial modeling and computer simulation techniques to quantify the microbiological hazards associated with certain processes used in the food industry. These techniques clearly are of value in establishing critical limits (control parameters for CCPs) on a more scientific basis, and for evaluating the performance and equivalence of HACCP plans.[16,22,52,65]

1.5 THE ROLE OF LEGISLATION

Food laws protect the consumer against fraud, adulteration, and contamination of food; they also protect the honest manufacturer and trader against unfair competition. Many Roman and Greek civil laws were aimed at protecting their peoples against such malpractices. Further, many food laws and statutes introduced in the Middle Ages and the Renaissance were particularly aimed at protecting the public from the use of false weights and measures and the sale of adulterated food.[36]

Modern food law dates back to the 19th century, and not surprisingly the first of such UK laws was the Adulteration of Food Act of 1860, which remains the foundation of UK food law.[64] Legislation concerning the hygiene of foodstuffs was developed somewhat later, with the first regulations being introduced at the turn of the 20th century. The regulations mainly concerned meat inspection in recognition of the large amount of illness resulting from the consumption of contaminated meat.

Hygiene legislation evolved throughout the 20th century and has included the regulation of important specific processing measures, such as the time and temperature requirements for the pasteurization of milk[48] as well as the introduction of general hygiene measures via general hygiene regulations within Food and Drug Acts and specific requirements in statutory codes.[37] Increasingly, it has been recognized that much of the existing national food legislation conflicts with requirements in other countries and thus leads potentially to nontariff barriers to trade. Further, many requirements in existing national and international hygiene legislation are based on perceptions of hygiene needs and value judgments of general control requirements rather than on a scientifically based assessment of need, based on an assessment of risk and identification of specific and quantifiable measures for the control of any unacceptable risk. Thus, there is much activity in reviewing existing legislation and implementing new legislation based more heavily on the scientific process of risk assessment and the identification of specific food safety objectives.[26] This change is reflected in the UK Food Safety Act 1990[64] and the ongoing review of the European Union hygiene regulations concerning animals and animal products. Changes are taking place throughout the world following the WTO/GATT Sanitary and Phytosanitary (SPS) Measures Agreement.[3] This follows the need, specified in the SPS Agreement, for member governments to promote the concepts of equivalency, transparency, and harmonization and the need to develop food control legislation using risk assessment techniques developed by international organizations such as the Codex Alimentarius Commission.[17]

Hygiene requirements in food legislation should be proportionate to real health risks and based on the best available knowledge of the public health risks associated with particular foodstuffs. Legislation should also be sufficiently flexible to accommodate changes in scientific knowledge, such as the identification of new foodborne pathogens, and advances in food technology, such as the development of new processing methods and food preservation techniques. Hence, legislation should contain only the objectives and principles for obtaining a safe food and should require industry to be responsible for demonstrably implementing appropriate control procedures that will ensure that these objectives and principles are fully met.[38]

Enforcement of hygiene legislation is an essential part of the activities of a food control agency. Much of current enforcement is probably ineffective for reasons described earlier in this chapter. The empowerment and proper training of enforcement officials in risk and hazard assessment procedures, along with basic knowledge and understanding of food microbiology and modern food processing technologies, are essential if such officials are to provide an effective control and advice function.

1.6 THE FUTURE

1.6.1 The Food Supply

According to United Nations statistics, the world's population increased from 2.5 billion in 1950 to 5.7 million in 1995 and will increase to 8.3 billion by 2025. Over approximately the same time period, the urban population increased from 17% of the population in 1950 to 35% in 1990 with an estimate to increase to 54% by the year 2020.[41] Much of the population growth and increased urbanization will take place in Third World countries. The absence of clean water and basic public health control measures, together with the poor housing and malnutrition associated with poverty in urban areas, will potentially lead to more foodborne and waterborne disease.[31] It has been estimated that the global food supply will have to increase by 75% over the next 30 years in order to feed the expected world population growth.[21]

The challenge to governments and food producers is to meet this food demand, recognizing that the majority of the demand will be for access to a safe and nutritious supply of food by Third World nations. This will only be achieved by the close collaboration of molecular biologists, economists, agronomists, food scientists, and food microbiologists. The molecular biologist's role will be to explore ways to improve the productivity of food crops, including the resistance to microbial infection and diseases. The role of the food microbiologist, in addition to improving food safety, will be to identify better ways to reduce spoilage losses in basic food commodities, particularly those arising during storage and transportation to the expanding urban areas within Third World countries. Current world food shortages could be reduced if current losses through microbial spoilage were significantly reduced, and if more cost-effective means were available to preserve and transport food where it is in excess to where need exceeds availability.

An adequate supply of safe drinking water is not available in many Third World countries. Such water will increasingly be required for agricultural and food processing in these countries and will put further pressure on water supplies. Similar concerns of water availability are present in Western countries, with the effects of global warming increasing these concerns. The development of internationally accepted procedures for disinfecting water for reuse in food processing is under consideration by the Codex Alimentarius Commission. There is a particular need to develop means of detecting reconditioning system failures because such failures can lead to catastrophic public health problems.

The demand among consumers in developed countries for "in-season" fruits and vegetables throughout the year has led to a large trade in fresh produce from Third World countries. This trade provides essential revenue for these countries and is expected to increase, as will further processing of such fruits and vegetables in the country of origin. However, as noted previously, such produce may be contaminated with a wide range of foodborne pathogens (Chapters 25 and 27), and effective means for preventing and eliminating such contamination are needed.[14] The transfer of food technology knowledge and microbiological expertise to Third World countries is needed for the production of microbiologically safe foods for home consumption and for distribution throughout the world.

The growth in the convenience foods market, particularly seen in the developed countries over the past 20 or so years, and reflecting the changing lifestyles of the peoples of these countries, is also being developed in an appropriate form in Third World countries. Examples of "hurdle technology" to preserve some of the traditional foods of these countries are discussed and described in Chapter 14. In Western countries, the demand is for such foods to appear to be less processed and to have a "fresh" and more natural appearance and taste. Thus, the trend is to use less preservatives in foods and to generally apply processes that cause less damage to the organoleptic characteristics of the food. Therefore, increased growth in the chilled and frozen food sectors is expected to continue. However, the energy demand for the storage of such foods is particularly high. As energy costs and the associated environmental concerns increase, the need for development and application of preservation technologies that enable good storability at ambient temperatures and give equal consumer satisfaction will increase. Such products are particularly relevant to meet the needs of Third World countries.

1.6.2 New Technologies

To meet the consumer demands described above, the food industry has explored, and continues to explore, the application of new technologies to preserve foodstuffs. Thus, there has been much research into processes that use less or no heat to commercially sterilize foods. Such developments include the use of electrical resistance (Ohmic) heating, high pressure, pulsed electric fields, and possibly even pulsed light and pulsed high intensity magnetic fields; these techniques are discussed further in Chapters 2 and 13.[28] Other research has centred on exploring the potential incorporation into foods of natural antimicrobial agents from plants and animal tissues and of such agents produced by microorganisms (Chapters 2, 12, and 13).

While the effectiveness of many of these developments has been demonstrated under experimental conditions in the laboratory, few have progressed to commercial application because of scientific limitations or because the available technology for their application had limitations. A potential further difficulty with many of these applications is the ability to demonstrate the degree of microbial lethality achieved. For instance, should high pressure sterilization become technically possible, what should be the basis for demonstrating to a regulatory authority that the process will ensure the same degree of microbiological safety as the current thermal process? There are several problems in demonstrating equivalency with two such processes. The first is the different microbial inactivation kinetics of the two processes and, second, their different effectiveness against specific pathogenic microorganisms.[28] It is arguable that traditional approaches to microbiological safety assurance are inadequate for the judgment of safety of new process with different inactivation kinetics, and that new rationales should be developed based on modern risk assessment procedures.

There is increasing awareness that there are interactions between microorganisms and foodstuffs resulting in microorganisms adapting to their environment, and that virulence and survival of pathogens can be directly affected by a particular preservation system.[6,47] Such interactions, their effects on gene expression, and their significance to the use of existing and new preservation technologies needs to be studied, ie, do they change our assessment of the degree of microbiological safety assurance provided by these technologies?[11]

Parallel with research into new preservation technologies have been developments in the design and the construction materials used for food processing equipment, allowing a plant to operate hygienically for long time periods and thus improving production costs. These development are considered in Chapter 59.

Other new technologies recently applied to improve the microbiological safety of foods include the incorporation of antimicrobial agents into food contact materials and the incorporation of sensors into packaging material that provide visual evidence of time and/or temperature abuse.

1.6.3 Foodborne Illness

The prevalence of foodborne pathogens and epidemiological knowledge of the extent, sources, and causative factors that lead to foodborne illness remain unknown in many parts of the world. Even in Western countries new agents transmissible through foods and capable of causing illness continue to be found. Many of these do not cause classic acute gastroenteritis but long-term chronic illness often of a serious debilitating nature. One of the most recent of these has been the demonstration that "new variant" Creutzfeldt-Jakob Disease (v-CJD) in humans is almost certainly caused by the consumption of beef contaminated with the prions that cause bovine spongiform encephalopathy (Chapter 57).

What other agents will become a concern as a result of changes in food consumption, production, and processing practices, or discovered as a result of the development of new detection methods (Chapters 61–64)? This cannot be predicted but there are a number of trends that will potentially increase the risk of foodborne illness. These include an increase in the numbers of persons in populations more susceptible to illness because of some deficiency in immune competence as a result of age, disease, or medical treatment (Chapter 55). In addition, there is the likely increase in susceptibility to foodborne illness of persons living in Western countries as a result of less contact with foodborne pathogens and, hence, less triggering of the immune system (illness may more frequently arise as a result of increased travel to Third World countries and/or increased importation of foods from Third World countries). Balancing these concerns is the recognition of these risks and the wider use of labeling and targeting food consumption advice at the "at-risk" consumer groups and travelers, together with the wider influence of bodies such as the FAO and WHO sponsoring training courses on HACCP and food control procedures throughout the world.

There is a need for increased surveillance of foods in all countries to determine the prevalence of foodborne pathogens and to identify potential new public health concerns that may require the introduction of new legislation or some other form of control. One particular concern that requires concerted international action arises from the increasing recognition of antibiotic-resistant organisms in the food chain and the potential for these organisms to transfer such resistance to clinically important microorganisms.[23,30,50] Such information, together with all epidemiological information on foodborne illness, should be collected and collated on a world basis[67] and disseminated via the Internet.

1.7 CONCLUDING REMARKS

It is of concern that despite the vast accumulation of knowledge of the microbiology of food spoilage and foodborne pathogenic microorganisms and our ability to document this knowledge in a book such as this, about one-third of the world's food produce is lost annually as a result of microbial spoilage, and a vast amount of microbially caused foodborne illness continues to occur throughout the world. There can be no doubt that there have been significant advances in our ability to produce microbiologically safe and stable foods, and that for the most part, foodborne illness and food spoilage are preventable. So why are we so unsuccessful? What appear to be lacking are sufficiently strong incentives to apply our existing knowledge to those activities and to those parts of the food chain where these would be most effective. Thus, if we want to significantly reduce foodborne illness, we must reduce the presence of the main causative agents, ie, *Salmonella* and *Campylobacter* species, in our food animals. If we want to prevent spoilage, we should store our foods under conditions that prevent their deterioration. Why do we not apply these preventive measures? The main reason is undoubtedly the cost benefit of applying the currently available methods. The challenge to food microbiologists in the 21st century is to identify control measures that are practical, widely applicable, and cost-effective. It is then up to governments to ensure that these measures are implemented and enforced.

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2

Strategies for Food Preservation

Grahame W. Gould

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2.1 INTRODUCTION

Most foods lose some degree of quality and shelf life following harvest, slaughter, or manufacture, depending on the type of food, its composition, formulation, processing, packaging, and conditions of storage. Loss of quality and shelf life may occur at any stage from the acquisition of raw materials to the eventual consumption of the finished product. These stages include storage, handling and assembly of ingredients, processing, packaging, distribution, retail display, transport, storage, and preparation for consumption. The stages, under varying degrees of control, are designed to provide a shelf life that ensures the safety and high quality of the finally consumed product.

The principal reactions that cause loss of food quality are well known. Consequently, they are the major targets for effective preservation, the prevention of quality loss in all its forms. Quality loss reactions are relatively few in number, and include physical, chemical, enzymic, and microbiological[50] (see Table 2-1).

When preservation fails and one of these reactions accelerates or escapes control, there may be a wide range of consequences. At the one extreme these may be trivial, though undesirable, changes in color, flavor, or texture of the food. At the other extreme, the most serious forms of quality loss are those associated with the presence or multiplication of certain microorganisms. These range from those that cause

Table 2-1 Major Reactions Leading to Quality Loss of Foods

Microbiological	*Enzymic*	*Chemical*	*Physical*
Growth or presence of toxinogenic microorganisms	Hydrolytic reactions catalyzed by lipases, proteases, etc	Oxidative rancidity	Mass transfer, movement of low molecular weight (MW) compounds
Growth or presence of infective microorganisms	Rancidity catalyzed by lipoxygenases	Oxidative and reductive discoloration	Loss of crisp textures
Growth of spoilage microorganisms	Enzymic browning	Nonenzymic browning	Loss of flavors
		Destruction of nutrients	Freeze-induced structural damage

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various degrees of spoilage, which nevertheless may result in economically important losses of foods, to the most hazardous of the food poisoning microorganisms that cause life-threatening diseases (Table 2-2). While most preservation techniques aim to control all the forms of quality loss, the overriding priority is always to minimize the occurrence and the growth of microorganisms, particularly those that cause food poisoning.

Table 2-2 Major Consequences of Quality Loss of Foods

Nature of Quality Loss	*Consequence of Quality Loss*
Presence of toxins Presence of pathogenic microorganisms	Hazard to the consumer
Microbial spoilage	Loss of food Limitation of shelf life
Potential microbial hazard	Inability to distribute and market product Product recall
Unacceptable rate of oxidation Unacceptable change in texture Development of rancidity Color change Flavor loss Texture change Poor keepability due to changes in color, flavor, texture	Increased packaging and distribution costs Limitation of shelf life Poor quality of marketed food

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The various forms of microbiological risk are controllable to a large degree by a range of preservation techniques, most of which act by preventing or slowing down microbial growth (eg, chilling, freezing, drying, curing, conserving, vacuum packing, modified atmosphere packing, acidifying, fermenting, or adding preservatives). A much smaller number of techniques act by killing microorganisms (eg, pasteurization and sterilization by heat and, to a much lesser extent, by ionizing radiation). Complementary techniques restrict the access of microorganisms to processed products (eg, clean handling, aseptic processing, and packaging).

A major trend is that new and "emerging" preservation techniques act primarily by inactivation (eg, ultrahigh pressure; high voltage electric pulse treatments; combined heat, ultrasonication, and pressure; the addition to foods of bacteriolytic enzymes).[10,27,40] A further trend is the development of higher quality products that are less "heavily" preserved, more natural, freer from additives, and nutritionally healthier. This trend results mainly from the food industry's reactions to the changing lifestyles of consumers (Exhibit 2-1).

Less severe preservation procedures are therefore being developed that make increasing use of (1) preservative factors in combinations (hurdle technology) in order to deliver less damage to product quality; (2) new methods of heating that are better controlled and therefore deliver milder heat to products; (3) combinations of precooking and refrigeration that produce foods with high quality and

Exhibit 2-1 Trends in Consumer Requirements and Food Industry Reactions

Trends in consumer requirements

- Improved convenience
  - —in preparation, storage, shelf life
- Higher quality
  - —in flavor, texture, appearance
- Fresher
- More natural
  - —with fewer additives
- Nutritionally healthier
- Minimally packaged
- Safer

Food industry reactions

- Milder processing
  - —minimal overheating
  - —less intensive heating
  - —nonthermal alternatives to heat
- Fewer additives
  - —less "chemical" preservatives
- Use of "hurdle" technologies or "combination preservation" systems
- Evaluation of natural antimicrobial systems as food preservatives
- Less use of salt, saturated fats, sugar; more low-calorie foods
- Reduced, environmentally friendly packaging
- Elimination of food poisoning microorganisms

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extended shelf lives; (4) modified atmosphere packaging to retain quality longer; and (5) use of more natural antimicrobial systems.

Many of the existing and emerging preservation techniques were derived empirically following many years of observation and research. However, more recently, new physiological investigations and "mode of action" studies are revealing that many of the most-used techniques act by interfering with the homeostatic mechanisms that microorganisms have evolved in order to survive extreme environmental stresses.[92] An understanding of the bases of the stress reactions is encouraging the development of more soundly based preservation procedures, aimed at increased control of the microorganisms that may cause foodborne disease or bring about the spoilage of foods.[43]

2.2 MICROBIAL TARGETS FOR FOOD PRESERVATION

While the number of microorganism types that cause food poisoning is relatively small, many types can cause spoilage. The principal food poisoning microorganisms, dealt with in detail in the various chapters of this book, are listed in Table 2-3 to indicate their relative resistance to heat and their ability to multiply at low temperatures. The major food spoilage microorganisms are not dealt with as specific genera or species in this book but rather, are discussed in the chapters dealing with particular food products and commodities. See also other major texts.[12,26,35,52,74] In Table 2-4 the most important of the spoilage organisms are categorized in such a way as to highlight not only their taxonomic relatedness but also, more importantly with regard to spoilage, the physiological and biochemical characteristics that govern their interactions with, and effects on, foods.

2.2.1 Gram-negative, Catalase-Positive, Oxidase-Positive Rods

Gram-negative, catalase-positive, oxidase-positive rods include the aerobic bacteria that grow rapidly and tend to dominate the spoilage flora of fresh meat,[15] fish,[46] poultry, eggs, and other high pH/high water activity (a_w), protein-rich foods and vegetables.[56] These bacteria grow in the presence of oxygen, at chill and at ambient temperatures. They include many psychrotrophs, such as some *Pseudomonas* species, that are able to multiply below 0°C. They are able to grow in simple media and are characterized by their ability to metabolize a wide range of organic compounds. With few exceptions, they are not particularly tolerant of extreme environmental conditions so that, for example, slight reductions in water activity (eg, in cured meats) or reductions in pH (eg, in marinaded fish products) tend to prevent their growth and encourage more tolerant groups (see below). *Acetobacter* oxidizes ethanol to acetic acid in alcoholic beverages and souring fruits. Others in this group (eg, *Gluconobacter*) may cause problems by producing haze and by oxidizing glucose to gluconic acid in soft drinks. Improvements in handling and bottling technologies have resulted in significant reductions in residual oxygen levels such that problems caused by these strict aerobes have become uncommon.[53]

Most of the microaerophilic *Campylobacter* species grow optimally between 40° and 42°C and not below 30°C. *Arcobacter* species and some campylobacters

Table 2-3 Food Poisoning Microorganisms of Major Concern

Minimum Growth Temperature	*Heat Resistance*	
	Low[*]	*High*[†]
Low (0°–5°C or so)	*Listeria monocytogenes* (INF)[‡] *Yersinia enterocolitica* (INF) *Aeromonas hydrophila* (INF)	*Clostridium botulinum* E and nonproteolytic B (TOX)[§] *Bacillus cereus* (INF and TOX) *B. subtilis* (TOX) *B. licheniformis* (TOX)
Medium (5°–10°C or so)	*Salmonella* species (INF) *Vibrio parahaemolyticus* (INF) *Escherichia coli* enteropathogenic and verocytotoxigenic strains (INF) *Staphylococcus aureus* (TOX)	
(10°–15°C or so)		*Clostridium botulinum* A and proteolytic B (TOX) *Clostridium perfringens* (INF)
High (over 30°C)	*Campylobacter jejuni* and *C. coli* (INF)	

[*] In excess of a 6-log inactivation of vegetative microorganisms by pasteurization, eg, at a temperature of about 70°C for 2 minutes.

[†] In excess of a 6-log inactivation of spores at temperatures ranging from about 90°C for most heat-sensitive types to about 120°C for 10 minutes for the most heat-tolerant types.

[‡] INF: organisms that may contaminate foods and may multiply in them, and which cause food poisoning by infection.

[§] TOX: organisms that may contaminate foods and multiply in them to form toxins that then cause food poisoning by intoxication.

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are unable to grow at 40°C and may multiply below 25°C.[9,91]

2.2.2 Gram-negative, Catalase-Positive, Oxidase-Negative Rods

The fermentative organisms in this group include the enterobacteriaceae which, apart from the important pathogenic species, contain others that commonly contaminate and grow on many raw foods, particularly if they are stored warm. However, some, such as many of the *Erwinia carotova* group, that characteristically cause plant diseases (eg, soft rots, wilts, and necroses), can grow at temperatures down to about 5°C. As a group, these organisms are not characterized by particular resistance to any of the environmental extremes typical of some food preservation procedures.

2.2.3 Gram-positive, Catalase-Positive, Nonsporing Rods

The corynebacteria, which include more than 30 species, are often pleomorphic, with granulated and club-shaped cells. Many are mesophilic and normally inhabit the skin of man and other animals. However, some that are psychrotrophic are common on plant materials, so that they usually contaminate vegetables and other foods. Their relative tolerances to some preservatives, such as sulfite, encourage the dominance of some members of this group (eg, *Brochothrix thermosphacta*) in the spoilage flora of products such as UK breakfast sausage. Resistance to carbon dioxide, and the ability to grow in low-oxygen environments, encourages the growth of organisms of this group in modified atmosphere and vacuum packed, chill-stored meat.[15]

2.2.4 Gram-positive, Catalase-Positive Cocci

The Gram-positive cocci include many organisms that are commensal on the skins of animals, and are therefore widespread. The aerobic micrococci and facultatively anaerobic staphylococci are often found together in milk products and, being a generally salt-tolerant group, tend to be selected in high-salt cured meat products. In vacuum- or oxygen-free modified atmosphere packs, the micrococci may be

Table 2-4 Food Spoilage Microorganisms of Major Concern

	Microorganisms	
Characteristics	*Examples*	*Foods Typically Affected*
Gram-negative, catalase-positive, oxidase-positive rods	Obligate aerobes: *Pseudomonas, Alkaligenes, Moraxella, Alteromonas, Flavobacterium*	Protein-rich foods (fish, meat, poultry) and vegetables at chill temperatures
	Shewanella, Photobacterium	Fish
	Acetobacter, Acetomonas	Alcoholic beverages
	Gluconobacter	Soft drinks
Gram-negative, catalase-positive, oxidase-negative rods	Fermentative: Enterobacteriaceae, *Escherichia, Citrobacter, Klebsiella, Erwinia, Proteus* Nonfermentative: *Acinetobacter, Xanthomonas*	Most raw proteinaceous foods of animal and plant origin
Gram-positive, catalase-positive, nonsporing rods	Coryneform bacteria: *Corynebacterium, Kurthia, Arthrobacter, Brochothrix*	Cured meats and sausages Fresh meats in vacuum or low-O_2 "modified atmosphere" packs Vegetables
Gram-positive, catalase-positive cocci	Aerobes: *Micrococcus* Facultative anaerobes: *Staphylococcus*	Milk products Cured meat products, especially if high salt
Gram-positive, catalase-negative nonsporing rods	Facultatively anaerobic, homofermentative, and heterofermentative species of *Lactobacillus*	Milk and milk products, fermented dairy, meat and vegetable foods, meat in vacuum, raised CO_2, and/or low-O_2 packs
Gram-positive, catalase-negative cocci	Facultatively anaerobic lactic acid bacteria: *Streptococcus, Lactococcus, Enterococcus Leuconostoc, Pediococcus*	Cured meats, particularly if low salt Dairy products
Gram-positive, catalase-positive, spore-forming rods	Aerobic or facultatively anaerobic species of *Bacillus*	Heated foods with pH values above about 4
Gram-positive, catalase-negative, spore-forming rods	Strictly anaerobic species of *Clostridium*	Heated foods with pH values above about 4.5 (4 for *C. butyricum*) and with restricted availability of oxygen
Yeasts	Mostly facultative anaerobes: *Saccharomyces, Candida, Zygosaccharomyces, Torulopsis*	Low pH and low a_w foods (drinks, jams) whether or not oxygen is excluded
Molds	Mostly strict aerobes: *Penicillium, Aspergillus*	Low pH and low a_w foods exposed to air

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suppressed, but some may utilize nitrate as an alternative to oxygen as an electron acceptor and, therefore, continue to grow under these conditions in nitrate-containing foods. Staphylococci will be selectively encouraged if storage is well above normal chill temperatures. Some strains are sufficiently thermoduric to survive commercial procedures for milk pasteurization. *Deinococcus* and *Deinobacter* species are unique in being more radiation resistant than bacterial spores.[72] *Deinococcus radiodurans* is one of the most radiation-resistant bacteria known and is also relatively resistant to low a_w conditions. It was originally isolated from irradiated ground beef. Of the three other species in this genus, *D. radiopugnans* was isolated from irradiated haddock, *D. radiophilus* from irradiated Bombay duck, and *D. proteolyticus* from llama feces.[71] The only species of *Deinobacter, D. grandis*, was isolated from elephant and carp feces.[81] While surviving most of the low dose applications of radiation that are currently employed, these organisms have not proved to be a problem in current food irradiation practice.

2.2.5 Gram-positive, Catalase-Negative, Nonsporing Rods and Cocci

The lactic acid bacteria are characterized by their homofermentative or heterofermentative modes of metabolism, with the production of lactic acid and consequent souring, or souring plus the formation of gas, in spoiling foods. They are widespread, particularly in the flora of fermentable natural materials such as milk, fruit juices, vegetables, and silage. They often tend to be enriched in foods in which the Gram-negative floras mentioned above (Sections 2.2.1 and 2.2.2) have been inhibited, eg, by the removal of oxygen, by an increase in carbon dioxide, or by a slight reduction in water activity. They are generally more tolerant of low pH than other spoilage bacteria, some continuing to multiply at pH values as low as 3.6. Lactic acid bacteria are common beer spoilage organisms.[53] Some pediococci are extremely salt tolerant. Growth of lactic acid bacteria in many foods is often organoleptically acceptable. They may sometimes play an important "fail-safe" role, ensuring that in foods stored incorrectly, particularly too warm for too long, the pH falls, preventing the growth of more undesirable microorganisms.[49]

2.2.6 Gram-positive, Catalase-Positive, Spore-Forming Rods

The *Bacillus* genus contains 48 species, including psychrophiles, mesophiles, and thermophiles.[11] The primary habitat of most *Bacillus* species is the soil. From there they become widely disseminated in the air and in dust and readily gain access to unprotected foods. Psychrotrophic, mesophilic, and thermophilic members of the genus produce spores that have low, medium, and high heat resistances, respectively (eg, *D*-values of a few minutes at about 80°C for some of the psychrotrophs, at about 100°–120°C for many of the mesophiles, and at well above 120°C for some of the thermophiles, such as *Bacillus stearothermophilus*). They include some strict aerobes, but also facultative anaerobes, so that they are capable of growth in foods in all forms of packaging. Their ability to sporulate ensures that they are of most concern in heated, cooked, or pasteurized foods, particularly if the pH is well above 4, since only a few can grow at pH values this low (eg, *Bacillus coagulans*, at about pH 3.8).

2.2.7 Gram-positive, Catalase-Negative, Spore-Forming Rods

The strictly anaerobic *Clostridium* genus contains over 90 species and, like *Bacillus*, includes thermophiles, mesophiles, and a small number of important psychrotrophs.[17,18] Many clostridia are highly proteolytic and therefore they putrefy food. However, there are important exceptions such as *C. butyricum* and *C. tyrobutyricum*, which cause spoilage by producing low odor threshold acids, and psychrotrophic strains of *C. botulinum*, which are highly saccharolytic. The thermophiles include some of the most heat-resistant spores known (eg, *Clostridium thermosaccharolyticum*). Like the thermophilic *Bacillus* species, these can cause spoilage of thermally processed foods distributed in hot climates unless specially increased heat processing has been applied or nisin has been included to prevent the outgrowth of surviving spores.[34]

2.2.8 Yeasts and Molds

The yeasts[23,32] and molds[31] are major spoilage organisms predominantly in low water activity foods (eg, jams, syrups, dried foods with insufficiently lowered water activities, dried/cured meats, and fish) and in foods in which the pH is low (eg, fruits) or pH has been reduced to levels below about 3.8 (eg, in pickles and marinaded products), because they are extremely tolerant of these two factors. Additionally, some yeasts, eg, *Zygosaccharomyces bailii*, are extremely resistant to weak acid preservatives such as sorbate and benzoate.

While many spoilage yeasts will grow in the presence or absence of oxygen, molds are generally inhibited in oxygen-free packaged foods and are more sensitive than yeasts to carbon dioxide. However, the levels of oxygen necessary to completely suppress mold growth may be very low and difficult to achieve under practical conditions unless some form of oxygen scavenger is employed. Some molds may continue to grow slowly and degrade even hermetically sealed foods. For example, *Byssochlamys* and *Talaromyces* species, which produce relatively heat-resistant ascospores (*D*-values of about 5 minutes and up to 200 minutes, respectively, at 80°C[29]), are able to grow at low oxidation-reduction potential (Eh) and cause liquefaction of pasteurized fruits or pickled vegetable products packed in cans or glass jars.

2.3 PRESERVATION AND SHELF LIFE EXTENSION

2.3.1 Basis of Food Preservation

First, food preservation is based on the delay or prevention of growth of food poisoning microorganisms and the spoilage types summarized above. It must therefore operate through those factors that most effectively influence the growth and survival of microorganisms, including freezing, storage at refrigeration temperatures, and the addition of preservatives. There are over 3,500 additives used in foods, many of which are derived from animal and plant materials. The additives include antioxidants, colors, emulsifiers, stabilizers, sweeteners, flavors, and preservatives. Relatively few types of additives are listed as preservatives. Additives listed as acidulants may also have preservative effects. Before additives are approved they must undergo extensive testing and evaluation to ensure that they are efficacious and toxicologically safe and to ensure that the incidence of allergic reaction is low (as low as between 3 and 15 people per 10,000, which is a lower incidence than allergic reactions to foods such as strawberries and shellfish). Most categories of additives, including preservatives, require approval by relevant regulatory authorities,[88] eg, in European Union (EU) countries[6] and in the United States,[7] a distinction is made between additives classified as GRAS (generally regarded as safe), prior-sanctioned, permanent, or permitted only on an interim basis.

Preservation factors include a number of physical factors, some chemical and some microbiological, ie, they depend on the nature of the microorganisms that are present. These factors have been categorized in a number of ways, but the categorizations that are most widely quoted[50,51,73] separate them into the following groupings (Table 2-5):

- ***Intrinsic factors,*** which include physical and chemical factors that are *within* a food, and with which a contaminating microorganism is therefore inextricably in contact.
- ***Processing factors,*** which include factors that are deliberately applied to foods in order to achieve improved preservation.

Table 2-5 Major Factors Affecting Microbial Growth and Survival in Foods

Intrinsic Factors				
Chemical	*Physical*	*Processing*	*Extrinsic Factors*	*Implicit Factors*
Nutrients	a_w, ERH	Changes in food composition	ERH during storage	Microbial growth rates
pH and organic acids buffering	Ice and freeze concentration	Changes in types of microorganisms	Temperature during storage	Synergistic effects (food components, microorganisms)
Oxidation-reduction potential	Changes in structure	Changes in numbers of microorganisms (heat treatments, irradiation)	Oxygen level, presence of other gases (CO_2 specifically)	Antagonistic effects (food components, microorganisms)
Antimicrobial substances	Changes in microstructure (emulsification)			

Note: a_w, water activity; ERH, equilibrium relative humidity.

Source: Data from D.A.A. Mossel, Essentials and Perspectives of the Microbial Ecology of Foods, in *Food Microbiology: Advances and Prospects, Society for Applied Bacteriology Symposium Series No. 11*, T.A. Roberts and F.A. Skinner, eds., pp. 1–45, © 1983, Academic Press, and D.A.A. Mossel and M. Ingram, The Physiology of the Microbial Spoilage of Foods, *Journal of Applied Bacteriology*, Vol. 18, pp. 232–268, © 1955, Society of Applied Microbiology.

- ***Extrinsic factors,*** which include factors that influence microorganisms in foods, but which are applied from *outside* the food and act during storage.
- ***Implicit factors,*** which include factors that are related to the nature of the microorganisms themselves, and to the interactions between them and with the environment with which they are in contact during growth.

To the above factors Mossel[73] additionally recognized:

- ***Combination effects*** (net effects), which take into account the fact that many of the factors strongly interact so that the overall effects of combinations of factors may not be readily predictable.

Potentially useful combination effects are increasingly derived from modern predictive modeling studies in which the effects of combinations of factors provide most of the data from which mathematical models are derived.[67–69]

2.3.2 Major Food Preservation Technologies

The major technologies employed to preserve foods are all based on a relatively limited set of factors, so that their range is necessarily limited also. The technologies are summarized in Exhibit 2-2 to highlight the fact that most of the techniques act primarily by slowing down, or in some cases completely arresting, microbial growth.

Exhibit 2-2 Major Existing Technologies for Food Preservation

Techniques that slow or prevent the growth of microorganisms
- Reduction in temperature
  —chill storage, frozen storage
- Reduction in water activity
  —drying, curing with added salt, conserving with added sugar
- Reduction in pH
  —acidification (eg, use of acetic, citric acids, etc), fermentation
- Removal of oxygen
  —vacuum or modified atmosphere packaging
- Modified atmosphere packaging
  —replacement of air with CO_2, O_2, N_2 mixtures
- Addition of preservatives
  —inorganic (eg, sulfite, nitrite)
  —organic (eg, propionate, sorbate, benzoate, parabens)
  —bacteriocin (eg, nisin)
  —antimycotic (eg, natamycin)
- Control of microstructure
  —in water-in-oil emulsion foods

Techniques that inactivate microorganisms
- Heating
  —pasteurization
  —sterilization

Techniques that restrict access of microorganisms to products
- Packaging
- Aseptic processing

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2.3.3 New and Emerging Food Preservation Technologies

The newer and still-developing techniques include more natural approaches, eg, modified atmosphere packaging, use of "protective cultures," and use of bacteriocins, other culture products, and enzymes (Exhibit 2-3). It is interesting that, in contrast to the more traditional techniques, most of the newer ones that are being explored act by inactivation, eg, irradiation; the application of high hydrostatic pressure; high voltage electric discharge (electroporation); ultrasonication combined with increased temperature and slightly raised pressure (manothermosonication); and addition of bacteriolytic enzymes (eg, lysozyme; Exhibit 2-3).

2.4 COMBINATION PRESERVATION TECHNIQUES

The major preservation techniques that prevent or delay microbial growth are reduction in temperature, reduction in pH value, reduction in water activity, and the application of heat, which inactivates microorganisms. However, these and other techniques are increasingly used together in combination or "hurdle " technologies[59,60,90] (Chapter 14). It is widely expected that these approaches will find increasing future application. While the development of many of the most-used combinations in the past was empirical and traditional in different regions of the world, the basis of their efficacy has been uncovered in some instances, as illustrated below, and this is leading to a more rational approach to preservation.

2.4.1 Low pH–Weak Acid Synergy

Among the most widely used traditional preservation treatments are those in which an antimicrobial

Exhibit 2-3 New and Emerging Technologies for Food Preservation

Natural additives
- Animal-derived antimicrobials
  - —lysozyme
  - —lactoperoxidase system
  - —lactoferrin, lactoferricin
- Plant-derived antimicrobials
  - —herb and spice extracts
- Microbial products
  - —nisin
  - —pediocin
  - —other bacteriocins and culture products

Physical processes
- Gamma and electron beam irradiation
- High voltage electric gradient pulses (electroporation)
- High hydrostatic pressure
- Combined ultrasonics, heat, and pressure (manothermosonication)
- Laser and noncoherent light pulses
- High magnetic field pulses

acid is used as an additive or generated by fermentation, and its effectiveness is enhanced by lowering the pH value. Indeed, the majority of the useful food preservatives fall into this category. A few wide-spectrum food preservatives remain, which are effective at pH values near neutrality (Table 2-6; Chapter 9). The acids include the inorganic preservatives, sulfite and nitrite, and the weak organic acids. The organic acids generally increase in effectiveness in the order acetic, propionic, sorbic, benzoic (Chapter 8), and this reflects their increasing lipophilicity. This gives a clue to one element of their modes of action, because it is the lipid solubility, mainly of their undissociated forms, that enables them to cross the microbial cell membrane and gain access to the cytoplasm within the cell[13,14] (Chapter 8).

The second element important in their modes of action is the dissociation constant of the acid, because it is the undissociated form that is most lipophilic and, therefore, most readily permeates the membrane. The pH value and the dissociation constant determine the proportion of acid that is in this form. The p*K* values of the common weak acid preservatives range from 4.2 (benzoic) to 4.87 (propionic), so that at pH values much above these, antimicrobial activity is much reduced (Chapter 8). At the pH values of most foods, microorganisms maintain an internal pH that is higher than that of their surroundings. On entering the cytoplasm, the undissociated acids therefore tend to dissociate into hydrogen ions and the particular anion. The additional hydrogen ion may be exported by the cell to maintain the high internal pH, but this is energy demanding so that cell growth may be restricted. If the en-

Table 2-6 Most-Used Food Preservatives

Preservatives	*Examples of Foods in Which They Are Used*
Weak lipophilic organic acids and esters	
Sorbate	Cheeses, syrups, cakes, dressings
Benzoate	Pickles, soft drinks, dressings
Benzoate esters (eg, methyl, propyl)	Marinaded fish products
Propionate	Bread, cakes, cheese, grain
Organic acid acidulants	
Acetic, lactic, citric, malic, etc	Acidulants for low pH sauces, mayonnaises, dressings, salads, drinks, fruit juices, and concentrates
Mineral acid acidulants	
Phosphoric, hydrochloric	Acidulants, as above
Inorganic anions	
Sulfite (SO_2, metabisulfite)	Fruit pieces, dried fruits, wine, meat (sausages)
Nitrite	Cured meats
Antibiotics	
Nisin	Cheese, canned foods
Natamycin (pimaricin)	Soft fruit, dry-cured meats
Smoke	Meats and fish

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ergy demand is greater than that available, the pH of the cytoplasm will eventually fall to a level that is too low for growth to continue. In addition, careful uptake studies have shown that a minor part of the preservative actions of some of the acids, and of sorbic and benzoic in particular, results from effects additional to the membrane gradient-neutralizing ones mentioned above,[28] and from membrane dissipation effects that may be indirect (Chapter 8).

There is an important, rational, and practical conclusion to be drawn. Whenever possible, microbial growth is best prevented by simultaneous reduction of pH and the presence of a weak acid preservative and, because there is then an increased energy demand on the cell, by anything additional that restricts the efficient generation of ATP. Examples include the application of further energy-demanding stresses, and, for facultative anaerobes, the removal of oxygen in vacuum or modified atmosphere packages.

2.4.2 Modified Atmosphere Packaging Plus Chill Storage

Carbon dioxide is a weak acid that is widely used as a food preservative in some countries[30] (Chapter 10). When added to a hermetically sealed foodpack, it dissolves and comes into equilibrium within the food to form mainly bicarbonate anion and other chemical species according to the partial pressure of the gas, its relative volume, and the pH and buffering capacity of the food. The efficacy of carbon dioxide results from a number of unrelated phenomena. The elimination of oxygen found in some forms of modified atmosphere packaging alters the spoilage flora by preventing the growth of strict aerobes and slowing the growth of facultative anaerobes by restricting the amount of energy they can obtain from substrates. However, carbon dioxide has a strong specific antimicrobial action, which is very microorganism specific. For example, many oxidative Gram-negative bacteria such as species of *Pseudomonas* are sensitive to concentrations as low as 5%, while many lactic acid bacteria and yeasts are capable of growth in the presence of 100%, and even in carbon dioxide under pressure. Carbon dioxide-enriched packaging of foods such as fresh meats usually results in a shift of the spoilage flora from a rapidly growing Gram-negative to a slower growing Gram-positive association of strains.[22] In modified atmosphere packaged fresh fish, in contrast, the Gram-negative *Photobacterium phosphoreum* is very carbon dioxide tolerant and may therefore be selected as the dominant spoilage organism.[20]

Good low temperature control is vital for effective use of modified atmosphere packaging because the antimicrobial efficacy of carbon dioxide is *greatly* enhanced as the temperature is reduced. While the reasons for the synergism, deriving from the combination of CO_2 with low chill temperature, are not fully understood they probably include the increase in solubility of carbon dioxide that accompanies reduction in temperature.

In addition to the antimicrobial effects of modified atmosphere packaging, there are often important additional effects of the combination technique on food color. These effects together contribute to the useful extension of organoleptically acceptable shelf life. For instance, in the preservation of chill-stored fresh meat, carbon dioxide is usually employed at a concentration of 20% to 30%, with oxygen making up the remainder. While the raised level of carbon dioxide delays growth of the Gram-negative spoilage flora, the raised level of oxygen ensures that the haem pigments remain in the bright red oxymyoglobin form, which keeps the meat looking attractive to the purchaser and doubles the overall chill shelf life.[82]

2.4.3 Low Water Activity Plus Adjuncts

Reduction of water activity to values between about 0.65 and 0.86–0.90 in *intermediate moisture foods* (IMF), prevents the growth of most spoilage bacteria, depending on the pH value[61] (Chapter 7). However, many osmotolerant yeasts and molds multiply at these low water activities so that IMF are not inherently stable by means of their low water activities alone. However, addition of antimycotics, such as sorbic or benzoic acids, provides combinations that can control the osmotolerant fraction of the potential spoilage flora, resulting in long ambient shelf life, as in many semi-moist pet food products. However, these combination systems are less widely used for the preservation of human foods because of the organoleptic penalties resulting from the extensive drying or the high levels of solutes needed to sufficiently reduce the water activity. For this reason, combinations of reduced water activity and other adjuncts, including mild heating, have been successfully developed, because higher water activities, and therefore higher moisture levels and/or lower concentrations of solutes, may be used (Section 2.4.4).

2.4.4 Heat Plus Reduced Water Activity

Mild heating may inactivate nonspore forms of microorganisms in foods and injure surviving spores at temperatures well below those necessary for sterilization (Chapter 3). Lowered water activity is one of many potentially useful synergies of heat with other stresses that may be imposed on microorganisms in foods.

At pH values of 4.5 and above, a "botulinum cook" would normally be required to ensure the safety of long ambient shelf life products, unless some other inhibitory factor was known to be operating (Chapter 3). In particular, in some cured meat products, such an additional factor is low water activity, sometimes with a reduction in pH value and the addition of nitrite. These combination preservation systems allow the production of a wide variety of so-called shelf-stable products (SSPs), which have long, safe ambient stability, and yet require only pasteurization heat treatments.[58,59] The best-known examples include meat products of the Italian Mortadella type. Many other alternative water activity–pH–mild heat process combinations have been reported. Some are employed empirically around the world (Chapter 14), but many others remain unexploited.

2.4.5 Heat Plus Reduced pH Value

The synergy of low pH with mild heating is the basis of the thermal processing of high acid foods, in which the pH, below 4.5, is low enough to prevent growth from spores of *Clostridium botulinum*. However, other mild heat–low pH combination treatments with potential for the preservation of long ambient shelf life foods have been described, though little used. For example, it has been shown that mild heating of spores at low pH values decreases their resistance to subsequent heating, even if the subsequent heating is at a higher pH value.[4] This occurred with all types of spores tested, including *C. botulinum*. It was proposed as a potentially useful procedure for reducing heating requirements for the sterilization of long ambient shelf life foods, but difficulties in designing suitable large-scale processes were such that the acid sensitization procedure has not been used commercially. The mechanism of the effect is partially understood. During the acid heating, hydrogen ions replace some of the spore-bound calcium and other cations to form so-called "H-form" spores, which have reduced tolerance to heat. The procedure is so efficient that virtually all of the calcium, which makes up about 2% of spore dry weight, can be removed from the spores of some species, eg, *Bacillus megaterium*, by this means.[65] When the pH is raised, re-equilibration occurs as hydrogen ions in the spore are replaced again by cations from the food. This process is sufficiently slow enough that heating can be performed while the spores are still relatively heat sensitive. With the advent of modern aseptic processing and packaging techniques for pumpable liquid foods, this sequential combination acid sensitization process would be easier to apply than when it was first proposed.

2.4.6 Mild Heating and Chill Storage

There has been a recent expansion in the use of the combination of mild heating of vacuum packaged foods with well-controlled chill storage, particularly for catering, but also retail. These foods have been termed refrigerated processed foods of extended durability (REPFEDs)[77,78] and include "Sous Vide" products (foods mildly heated within vacuum packs).[62] While the new processes deliver high quality products, they also present new challenges regarding the ensurance of microbiological quality and safety.[66] The success of the procedures results primarily from the inactivation of the vegetative microbial flora by the mild heating, and also from the fact that the spores of psychrotrophic bacteria, which can grow at low chill temperatures, are generally more heat sensitive than the spores of mesophiles and thermophiles, which cannot grow at these temperatures (Chapter 5). The minimal heat treatment necessary to inactivate psychrotrophic vegetative pathogens is generally accepted to be 70°C for 2 minutes[36] or 72°C for 2 minutes[76] or equivalent, to achieve a 6- or 8-log reduction in numbers, respectively, of *Listeria monocytogenes*. The mild heating destroys the vegetative pathogens and the cold-growing fraction of the potential spoilage flora, while the minimal thermal damage and often conditions of low oxygen tension ensure high product quality. If temperatures can be maintained below 3°C, shelf life can be long. To ensure safety if storage temperatures are above 3°C but below 10°C, more severe heat processes are necessary to ensure inactivation of spores of pathogenic spore formers (eg, in excess of 6-log inactivation of spores of psychrotrophic strains of *Clostridium botulinum*,[63,64,76,78] or some form of demonstrable intrinsic preservation of products is generally regarded to be necessary[2,5,45] (Chapter 41).

2.4.7 Heating Following Irradiation

Ionizing irradiation sensitizes bacterial spores to subsequent heating, though the converse is much less effective.[38] The magnitude of the effect varies with irradiation dose rate and is increased if the spores are additionally preincubated at low pH values, as described above. It has been suggested, but not proven, that the effect derives from radiation-induced decarboxylation of peptidoglycan in the spore cortex, which is known to play a role in maintainance of the low water content in the enclosed spore protoplast. In support of this idea, it was shown that the heat sensitizing effect of irradiation was reversed when the treated spores were suspended in solutions of high osmolality before they were heated. It was proposed that this removed water from the partially rehydrated spore protoplast by osmosis, so returning it to the original fully heat-resistant state.

Should the irradiation of foods continue to expand, the synergism of this combination system may be worth reexamination as a practical means for reducing thermal processing requirements and for minimizing heat damage to the nutritional and organoleptic properties of some foods, while retaining long, safe ambient shelf life. The synergism has already been shown to be of practical use in reducing the heat resistance of the spores of thermophilic bacteria in irradiated spices that are subsequently used as ingredients in thermally processed foods.[54] Legal restriction of radiation doses to <10 kGy in most countries that allow food irradiation has prevented the use of some such synergies except for some specific food ingredients (eg, herbs, up to 30 kGy in the US[93] (Chapter 4). However, recent World Health Organization (WHO) and International Atomic Energy Agency (IAEA) recommendations, which state that there should be no upper dose limit for the treatment of food with ionizing radiation, may encourage a reexamination of the potential benefits of combination techniques.

2.4.8 High Pressure Synergy with Mild Heat

With regard to the pressure sensitivity of microorganisms, an important division exists between the vegetative and the spore forms of bacteria. While many environmental factors greatly affect pressure tolerance (in particular the water activity[80,89]), the generalization still holds that vegetative forms are inactivated by pressures in the region of 300 to 500 MPa but spores of some species resist pressures well in excess of 1,000 MPa. For this reason, most current applications of high pressure technology for food preservation are for low pH foods in which spores are not a problem because, even if they survive, they are unable to grow (Chapter 13).

However, there is a strong synergism of pressure with heat such that the spores of some species can be inactivated, even at pressures as low as 100 or 200 MPa, if the pressure is raised at the same time as the heat is applied. The basis for the efficacy of this combination treatment is partly explained by the fact the pressure in this intermediate region can directly cause spores to germinate.[19] Having germinated, they are then sensitive to heat, and also to pressure if it is high enough. While the required pressure-heat combinations are currently too extreme for wide commercial application for the sterilization of high water activity-high pH foods, the addition of other adjuncts (hurdles) may well introduce a new series of pressure combination procedures that are effective for long shelf life food preservation in the future. Presumably, any such novel procedure would have to achieve at least a 12-log kill of spores of *C. botulinum*, and other pathogenic spore formers, to match the accepted safety levels for thermally processed foods.[40]

2.4.9 Ultrasonication with Pressure and Mild Heat

While high intensity ultrasonic radiation has been known for many years to inactivate vegetative forms of bacteria, yeasts, and molds by their physical destruction resulting from the effects of cavitation, spores are known to be much more resistant. Synergism of ultrasonic waves with hydrogen peroxide[3] and with heat[79] have been described. However, application of ultrasonic energy with heat and with a slightly raised hydrostatic pressure together (manothermosonication) has more recently been shown to be an effective combination that effectively reduces, by about 10°C or more, the temperature of heating necessary to achieve a particular kill of vegetative cells and of spores.[85] While the detailed mechanism by which inactivation of the microorganisms occurs is not known, the physical basis is thought to include the effect of pressure on the vapor pressure of water. This increases the effectiveness of cavitation and, in particular, reduces the fall off in cavitation efficiency that normally accompanies a rise in temperature (Chapter 13).

Currently, manothermosonication is laboratory or small-pilot scale, but may well have future potential as an alternative pasteurization or sterilization method for liquid foods. Its application for solid foods is less likely because of the difficulty of economic delivery of sufficiently high ultrasonic intensities into solid substrates.

2.4.10 High Voltage Electric Discharge

Substantial inactivation of vegetative cells of bacteria, yeasts, and molds can be achieved by delivering pulsed electric discharges to liquid foods.[87] A major part of the mechanism of action is the puncturing of the cell membrane at the poles of the cell with respect to the voltage gradient.[86] This damage occurs because, at the high voltage gradients used, a potential difference of about 1 volt or more becomes applied across the cell membrane, which represents an enormous voltage gradient. The technique therefore acts by breaching one of the most important cell structures involved in many key homeostatic mechanisms, eg, control of cytoplasmic pH, maintenance of chemiosmotic ion gradients, and operation of osmoregulation mechanisms (Chapter 13).

2.4.11 Naturally Occurring Antimicrobial Systems

In addition to the more traditional and "emerging" systems employed to achieve desired safe shelf lives, there is consumer pressure for still milder, more natural means for the preservation of foods. This pressure has resulted in concentrated attention on the wide range of extremely effective naturally occurring antimicrobial systems seen in animals, plants, and microorganisms,[44] with the goal of exploiting these systems in foodstuffs[21,25] (Table 2-7).

Animal-derived systems include enzymes such as lysozyme, lactoperoxidase, other proteins such as lactoferrin or lactoferricin derived from lactoferrin by limited proteolysis, ovotransferrin, serum transferrins, small peptides such as histatins and magainins, and even the immune system. Plant-derived systems include phytoalexins, low molecular weight components from herbs and spices, phenolics such as oleuropein from olives, and essential oils. Microorganism-derived systems include organic acids, hydrogen peroxide, diacetyl and other low molecular weight substances, and bacteriocins such as nisin and pediocin and many others discovered in the last few years[1] (Table 2-7 and Chapter 12).

While many of these naturally occurring systems have been described and explored for food use, very few are actually exploited at this time. Only lysozyme, used to prevent defects in some types of cheeses caused by *Clostridium tyrobutyricum*,[16] and nisin,[24] used to help to preserve some cheese products and to protect some canned foods from spoilage by thermophilic spore formers, are in relatively wide use. However, the future potential for application of such systems is substantial as their efficacy is demonstrated in combinations with the other antimicrobial factors and procedures that can already be used to preserve foods.[25]

2.5 CONCLUDING REMARKS

2.5.1 Interference with Homeostasis As a Basis for Improved Food Preservation

While the range of well-established, new, and emerging techniques for food preservation and for

Table 2-7 Naturally Occurring Antimicrobial Systems

Origin	*Example*
Animals—constitutive systems	Myeloperoxidase in phagosomes Transferrins in serum Lactoperoxidase, lactoferrin in milk Lysozyme, ovotransferrin (conalbumin), avidin in eggs
Animals—inducible systems	Antibodies, complement in immune systems Attacins, cecropins in insects Magainins in frogs
Plants—constitutive systems	Eugenol in cloves, allicin in garlic, allyl isothiocyanate in mustard, oleuropein in olives
Plants—inducible systems	Low molecular weight (MW) phytoalexins, high MW polyphenolics in injured or infected plants
Microorganisms	Nisin, pediocin, and other bacteriocins from lactic acid bacteria Antimycotics from fungi (natamycin) Bacteriophages Yeast "killer toxins" Organic acids and other low MW metabolites

Source: Adapted with permission from G.W. Gould, Methods for Preservation and Extension of Shelf Life, *International Journal of Food Microbiology*, 33, p. 60, © 1996, Elsevier Applied Science, with data from V.M. Dillon and R.G. Board, eds., *Natural Antimicrobial Systems and Food Preservation*, © 1994, CAB International, Wallingford, Oxon.

achieving a satisfactory shelf life may seem to be diverse, there are two underlying themes that are fundamental to most of the newer, most successful methods. First, from the practical standpoint, many of the techniques are milder and less damaging to product quality and, in reaction to changing consumer requirements, are sometimes more natural than before. Second, from the scientific standpoint, clearly most of the techniques are effective because they overcome the various homeostatic mechanisms that microorganisms have evolved in order to survive extreme environmental stresses (Table 2-8).

There is a logic to many of the combination preservation procedures that are increasingly employed and researched because they act by amplifying this interference with homeostasis.[41] For example, reduction of a_w and pH interferes with macromolecular structure and assemblies and puts extra energy demands on cells, eg, for the maintenance of cytoplasmic pH. Addition of a weak organic acid delivers H^+ into the cytoplasm, further increasing the energy demand. Removal of O_2 then restricts the amount of energy available to perform some of these homeostatic responses, suggesting a rational basis for the efficacy of even such complex combination preservation mixtures as low a_w + low pH + organic acid + vacuum or modified atmosphere packaging.

2.5.2 Stress Responses of Microbial Cells

Many of the preservation systems that act to inhibit microbial growth are effective because they interfere with homeostasis by imposing stresses on microbial cells. However, the cells' responses to stress may result in an increased tolerance to that stress and even to other, apparently unrelated, stresses as well.[33,47,48] In pathogens, the stress response may include an increase in virulence.[70] Naturally occurring strains, eg, *Escherichia coli* and *Salmonella enteritidis*,[57] include a much higher incidence of high-rate mutators than laboratory-attenuated strains. This may lead to enhanced recombination among species and encourage the rapid emergence of antibiotic resistance and the distribution of virulence genes, for example, within the prokaryotic community.[57] Care must therefore be exercised in the development of milder preservation techniques in case situations in which microorganisms more easily, and less safely, overcome the applied stresses.[55] On the other hand, Archer[8] has pointed out that milder preservation procedures may, by delivering less extreme stresses to microorganisms, actually lead to a reduction in microbial stress responses in foods and, therefore, to an improvement in food safety.

Table 2-8 Active and Passive Homeostatic Mechanisms in Microorganisms

Environmental Stress	*Example of Homeostatic Reaction*
Active homeostasis	
Low nutrient levels	Nutrient scavenging, oligotrophy, generation of "viable nonculturable" forms
Low pH, presence of weak organic acids	Extrusion of hydrogen ions, maintenance of cytoplasmic pH, and membrane pH gradient
Reduced water activity	Osmoregulation, avoidance of water loss, maintenance of membrane turgor
Low temperature—growth	Membrane lipid changes, cold shock response
High temperature—growth	Membrane lipid changes, heat shock response
High oxygen levels	Enzymic protection from oxygen-derived free radicals
Biocides and preservatives	Phenotypic adaptation and development of resistance
Ultraviolet radiation	Excision of thymine dimers and repair of DNA
Ionizing radiation	Repair of DNA single strand breaks
Passive homeostasis	
High temperature—survival	Low water content in the protoplast of bacterial spores
High hydrostatic pressure—survival	Low spore protoplast water content?
High voltage electric dicharge	Low conductivity of spore protoplast
Ultrasonication	Structural rigidity of cell wall
High levels of biocides	Impermeable outer layers of cells
Population homeostasis	
Competition	Formation of biofilms, aggregates with some degree of symbiosis

Source: Adapted with permission from G.W. Gould, Homeostatic Mechanisms During Food Preservation by Combined Methods, in *Food Preservation by Moisture Control: Fundamentals and Applications,* G.V. Barbosa-Canovas and J. Welti-Chanes, eds., p. 401, © 1995, Technomic Publishing Company.

Most of the homeostatic mechanisms employed by microorganisms to overcome the effects of preservation procedures are *active*, ie, requiring changed gene expression and consequent changes in metabolism for their operation. However, some homeostatic mechanisms are *passive*, having been built in to the microbial cell when it was formed, ie, the *refractory homeostasis* of Gerhardt.[37] Low pH sensitizes bacterial spores to heat by overcoming a part of the spores' passive homeostatic resistance mechanism. High hydrostatic pressure and manothermosonication somehow do likewise. Electroporation interferes with all those homeostatic mechanisms that depend on an inbuilt, intact, semipermeable cytoplasmic membrane for their effective operation.[83] Altogether, therefore, interference with homeostasis, particularly by the logical use of combinations of techniques, remains an attractive focus for future work.

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3 Heat Treatment

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Appendix 3-A Definitions

3.1 INTRODUCTION

Heat treatment is used to produce safe and shelf-stable foods such as canned products and to eliminate pathogenic microorganisms, eg, the pasteurization of milk and liquid egg. Commercially canned foods that have undergone a satisfactory heat treatment may contain viable bacteria. For example, acid canned foods with a pH of 4.6 or lower (according to regulations in the United States (US)) or with a pH lower than 4.5 (according to regulations in the United Kingdom (UK)) may contain viable bacterial spores whose ability to germinate and give rise to growth is prevented by the acid pH of the food. Canned low-acid foods may contain spores of thermophilic bacteria that have a very high heat resistance but are unable to germinate and outgrow at temperatures below about 30°C. Such canned foods are stable in temperate climates but may spoil in tropical climates, necessitating either increased heat treatment or the use of antimicrobial additives. The term *commercial sterility* is often used to describe the process endpoint of canned foods. Strictly speaking, this term lacks meaning because sterility implies the absence of living organisms. In the canned foods industry, *commercially sterile* cans have received a process such that, for the specific food storage conditions, the food will neither spoil nor endanger the health of the consumer.[48]

While a patent for the preservation of food by heat was published in 1691 by Procter and White (quoted by Cowell[35]), practical food preservation by heat originated later. In about 1810, Appert demonstrated that heating foods in hermetically sealed containers could render them shelf stable for long periods at ambient temperatures.[7] Over a century later, Bigelow et al.[15]

made the first mathematical evaluation of heat sterilization applied to canned foods, and Ball[8] built on this to derive "methods of calculations for determining the time necessary to process canned foods." Many new approaches have since been proposed,[108] and processes are now set using elegant software programs and delivered by computer-controlled retorts or autoclaves. However, the original studies provide a sound foundation for almost all of the subsequent publications that underpin the modern thermal processing technologies. Even now, the Ball[8] method remains the basis of the methodologies most widely employed in the US, and Ball's method along with the method of Gillespy,[47] remains widely used in the UK.

The goal of thermal processing for commercial sterilization is to deliver sufficient heat to inactivate all of the potential spoilage and pathogenic microorganisms that may be present and capable of growth in a particular food. While this is the ultimate objective, as a result of the inactivation kinetics of microorganisms by heat, a more accurate definition is to reduce the probability of survival and/or growth of microorganisms in a particular food to an acceptably low level. The rationale of what is an acceptably low level has developed over many years and has centered on considerations of safety, following the original studies of Esty and Meyer[42] on the thermal inactivation kinetics of spores of proteolytic strains of *Clostridium botulinum.*

Early studies of the heat resistance of bacterial spores were made in order to develop processes for the production of canned foods. The concept of the *thermal death point* was used and was defined by Bigelow and Esty[14] as "the length of time at different temperatures necessary to completely destroy a definite concentration of spores in a medium of known hydrogen ion concentration." This was determined by sealing a suspension containing a known number of spores into tubes, immersing the tubes in a water bath at the required temperature for given periods of time after which the tubes were cooled rapidly and tested for sterility. Because the measured heat resistance of spores is influenced greatly by the nature of the suspending medium, studies of the resistance of spore crops in a standard phosphate medium at pH 7 were used as a reference prior to determination of their resistance in various foods. Based on a study of 109 strains of *C. botulinum,* Esty and Meyer[42] concluded that for the spores of this organism that showed the maximum heat resistance, heat treatments were necessary to sterilize suspensions containing billions of spores in phosphate buffer at pH 7 as follows: 4 minutes at 120°C; 10 minutes at 115°C; 33 minutes at 110°C; 100 minutes at 105°C; 330 minutes at 100°C. They also showed (1) that at 100°C the log of the number of viable spores decreased by more than 10 log cycles in a roughly linear manner with time of heating, (2) that germination of heated spores could occur after very long delays, and (3) that in juices from some canned foods, the heat resistance of the spores was greater than in the reference phosphate buffer.

Many subsequent experiments have been carried out to determine the heat resistance of spores of *C. botulinum* and *C. sporogenes* in phosphate buffers and in foods. The concepts of *F*-value and *z*-value were introduced, where *F* is the number of minutes required to destroy the organism in a specified medium, at a specified temperature, and *z* is the slope of the thermal death time curve expressed as the interval in temperature required for the line to pass through one log on semi-log paper.[107] The data of Esty and Meyer[42] for *C. botulinum* were recalculated incorporating corrections for heating and cooling lags. The resulting *F*-value for *C. botulinum* in low-acid canned food is 2.45 equivalent minutes at 250.0°F or 121.1°C with a *z*-value of 17.6°F or 9.8°C. This is recognized in the canning industry as the minimum practical *C. botulinum* process.[107] The safety aspects of the application of heat for food product sterilization are still based on these early studies. Esty and Meyer[42] had proposed standards for sterilization based on a reduction of spore populations by a factor of 10^{11} or 10^{12} and Hicks[53] argued that destruction of spores of *C. botulinum* by at least this extent was necessary to ensure the safety of low-acid canned foods. The generally observed efficacy of sterilization processes, and the lack of major problems when the processes are properly carried out, has provided evidence over many years that the basic rationale, however derived, is sound and cautious.[52,74,75,79,103]

The logarithmic death model as a function of a heat process, U, is $U = D(\log N_0 - \log N_U)$. U is the time of heating at a specified temperature, D is the time for a 90% reduction in the number of surviving organisms, N_0 is the initial microbial load, and N_U the endpoint survivor level (see Appendix 3-A for definitions of key terms); in microbial control processes it is a probability value such as 1 in 1,000 or 1 in 10^6 or 10^9. The value n is used to indicate the log-cycle reduction, $n = (\log N_0 - N_U)$, therefore, $U = n \times D$. In the food safety area, $n \times D$ is often used today as the process requirement, though it is a type of process design simplification and may be misleading. For example, a weakness of the $n \times D$ concept is that it only specifies a reduction in microbial load. Ideally, we should specify the endpoint in the microbial control process calculation. The endpoint will be the risk (expressed as a probability) that we are willing to take regarding having the pathogenic agent in a single serving of the food that we consume, but this requires an assumption about the initial numbers of microorganisms present, which is seldom confidently known. Examples of the use of $n \times D$ are: 12*D*, as the required *C. botulinum* process for low-acid canned foods; and 7*D*, to ensure safety with respect to the inactivation of salmonellae during the cooking of meat.[5] This latter use led to a US Department of Agriculture (USDA) requirement for this level of inactivation in the cooking of roast beef, and 5*D* was required for salmonellae in ground beef and *Escherichia coli* O157 in fermented sausages. Similarly, 6*D* processes (UK Department of Health[6]) or 7*D* processes[66] were proposed to ensure safety with respect to the survival of *Listeria monocytogenes* in mildly heated, in-pack pasteurized, chill-stored foods.

The ability to achieve these desired levels of destruction in particular processes depends absolutely on knowledge of the kinetics of heat inactivation of microorganisms in foods. This has been the focus of numerous research and modeling studies.

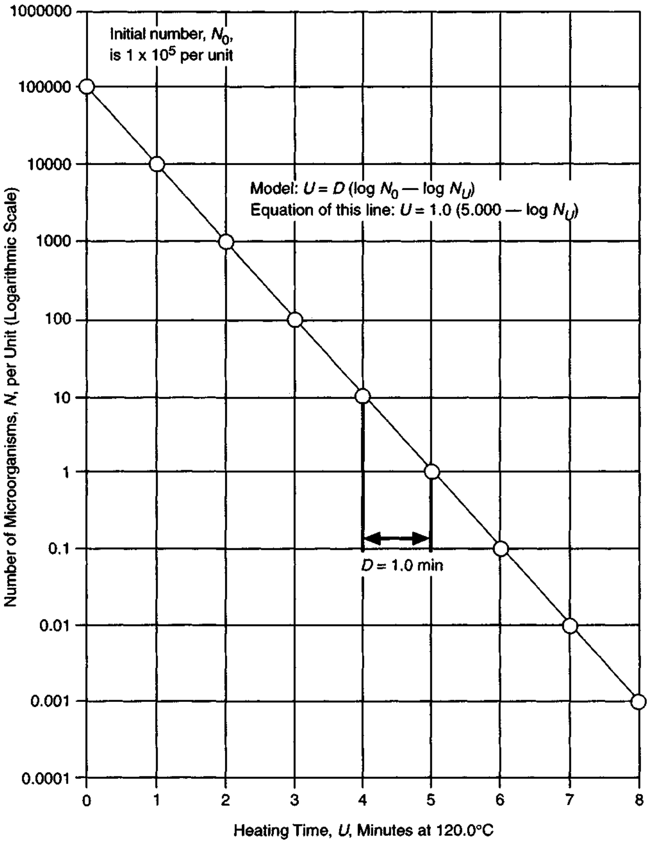

Figure 3-1 Basic Inactivation Kinetics. Microbial survivor data plotted on a semi-logarithmic graph. Number of survivors plotted on a log scale on the y-axis versus heating time on an arithmetic scale on the x-axis. *Source:* Copyright © 1995, I.J. Pflug.

3.2 KINETICS OF DESTRUCTION OF MICROORGANISMS BY MOIST HEAT

3.2.1 The Death Kinetics of One-Celled Organisms

Rahn[88] cited experiments showing that moist heat treatment at constant temperature resulted in a logarithmic rate of death of vegetative microorganisms and spores. Discounting various well-established deviations and artifactual effects that can influence the shapes of survivor curves (see below), the underlying kinetics is such that, when an aliquot of a homogeneous culture of microorganisms is heated at constant temperature, the numbers of survivors, therefore, represent a geometric progression, this condition is recognizable graphically by the straight line obtained by plotting the logarithms of their numbers against the time of exposure. In Figure 3-1, such data are plotted as log N versus heating time. When the logarithm of the number of surviving microorganisms is plotted on an arithmetic scale, as in Figure 3-1, the data points form a straight line and the y-axis is effectively expanded resulting in equal readability at all survival levels. This readability makes these graphs very useful in the microbial control field. The semi-logarithmic graph is immensely useful, but a nondistorted perspective of the effect of a constant stress on a homogeneous population must be kept in mind. This perspective can be obtained when the data in Figure 3-1 are plotted on a graph, with an arithmetic scale on both the x- and y-axes (Figure 3-2).

In the semi-logarithmic survivor curve model, the points on the graph larger than 1×10^0 correspond to survival levels larger than one microorganism per unit and all points less than 1×10^0 have a mean survival level less than one per unit, a fractional microbial survival level. There may be one surviving organism in 10 units, one in 100 units, one in 1,000 units, etc. as we approach zero organisms per unit.

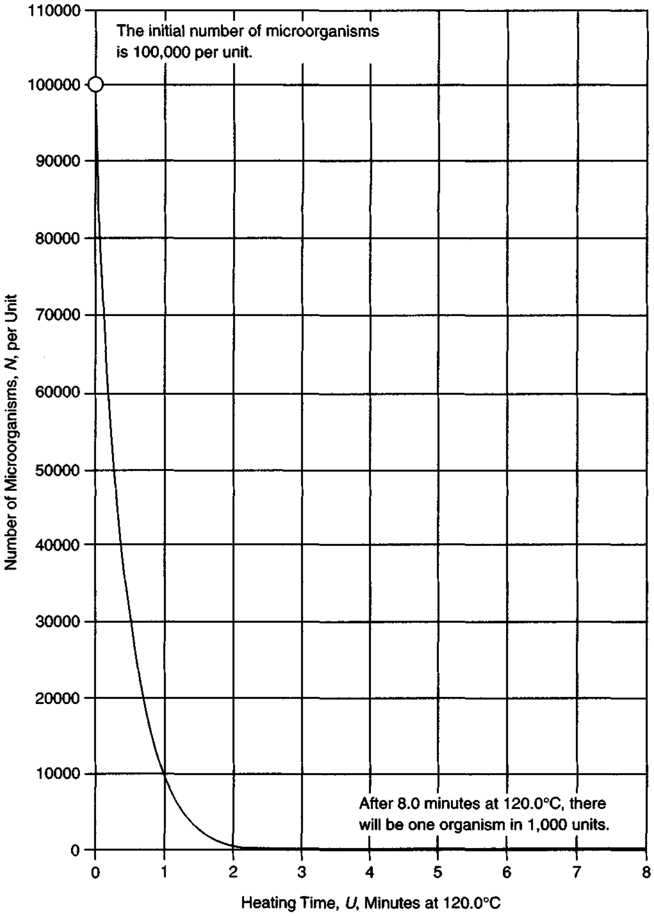

Figure 3-2 Basic Inactivation Kinetics. The same microbial survivor data as in Figure 3-1, plotted arithmetically on the y- and x-axes. *Source:* Copyright © 1995, I.J. Pflug.

These fractional survival levels are small positive numbers, for example, 1 in 10 = 0.1, 1 in 100 = 0.01, 1 in 1,000 = 0.001, etc.; however, their logarithms or exponential powers of 10 are the negative numbers: –1, –2, –3, etc. As we move toward zero survivors per unit, the survival fraction number becomes increasingly small and the negative exponent value increasingly large.

It has thus become customary to speak of "the logarithmic order of death" or the "exponential inactivation" of bacteria. Rahn,[88] however, did state in relation to plots of log survivors against time: "The difference in the order of death between bacteria and higher organisms has occasionally been questioned. A survey of all experiments on the order of death of bacteria until 1930[87] showed that 25% of the curves resembled those of multicellular organisms, 21% were strictly rectilinear, and 54% were concave upwards. Most of the 25% resembling multicellular organisms were actually obtained with 'multicellular bacteria,' ie, the bacteria were clustered. Since that survey, a number of investigations have verified the logarithmic order.[11,12,102,103,115] The difference between bacteria and higher organisms which causes this difference in kinetics of inactivation is the obvious one: with bacteria, the individual is dead when a single cell dies; with higher organisms, the death of one cell does not kill the organism."

The most commonly quoted hypothesis to account for the logarithmic order of death of microorganisms is therefore the thermodynamic one derived particularly by Rahn[88] and others.[26,31,64,75] The principal alternative follows from the proposals of Withell,[118] who pointed out that a distribution of resistances among individual cells in a population could lead to exponential inactivation kinetics if heat was assumed to act equally and uniformly on each cell. This "vitalistic" approach was developed by Cole et al.[34] to model the heat inactivation kinetics of *Listeria monocytogenes.*

The thermodynamic approach is based on the fact that at any instant, in any system at any temperature above absolute zero, there is a distribution of energy levels among the molecules that are present. In order to react with, and inactivate, a target molecule that is within a microorganism and is essential for viability, the energy of the reactant molecule must exceed a certain value. At constant temperature, therefore, individual target molecules in a population will all be subjected to the same *chance* of inactivation per unit time. At constant temperature the kinetics of inactivation will therefore be logarithmic. This is the simplest explanation of classical heat inactivation kinetics. It is attractive because it is soundly based on the true nature of heat. Also, it is important to remember that this simple explanation is necessarily based on some equally simple assumptions. The first (reasonable) one is that there is a distribution of energies among water or other molecules surrounding the "lethal target molecules" in bacterial cells. The second assumption is that all such target molecules have the same (in)activation energies. The third assumption, which is arguable, is that there is only one, or a small number, of such target molecules per cell (otherwise one would expect pronounced "shoulders" on survivor curves due to "multihit" kinetics). The fourth assumption is that substantial repair is not possible, otherwise, as with the inactivation by irradiation of bacteria, which have the capacity to repair damaged DNA, a shoulder would again be expected. With additional artifactual effects, eg, due to clumping of cells in suspension, nonuniform populations resulting from different ages of cells, there are therefore ample opportunities for deviations in practice. However, all are superimposed on the logarithmic order of death, which has a sound theoretical basis.

For example, Moats et al.[64] developed a model for the thermal death of bacteria on the basis that a cell has many critical sites, and that several of these sites must be inactivated before death occurs. The inactivation of individual sites was assumed to take place at random and the inactivation of each site followed first-order kinetics. Prokop and Humphrey[86] described a model for the inactivation of spores during heating that assumed that, following heating, a spore is in one of three sequentially occupied states: heat shocked, heat sensitive, or nonviable. A sequential first-order model was assumed, with sequential reaction rates, K_1 and K_2. (There is no evidence today that heat shock and heat sensitivity are linked.)

Several models for microbial destruction were developed by Brannen.[19] He discussed a multihit model in which nonlogarithmic survival is inherent in the organism. A similar model can be used to obtain all of the shapes of survivor curves shown in Figure 3-3.[18] Brannen[20] also investigated, via models, the consequences of the assumption that the genetic homogeneity and the DNA chemical homogeneity of a microbial population are not equivalent. Frederickson[45] described probabilistic microbial death models similar to those mentioned above. He observed that in such models, the extinction time of the "last" organism is a random variable, and that the modeled survivor curve shows sudden, unpredictable drops as the population size gets smaller. In studies where the logarithmic survivor curve was concave downward, Alderton and Snell[2] used a model in the form $(\log N_0/N)^a = Kt + C$ to analyze their data. Schalkowsky and Wiederkehr[94] developed a model in which they assumed that the incremental death time of heated microorganisms is directly proportional to prior exposure time and concluded that the logarithm of the survival time is normally distributed. They derived the normal distribution for survival times by assuming that prior exposure time does not produce a cumulative effect on the destruction process. Analysis of data showed an early normal distribution followed by a log normal distribution of survival times. The authors therefore indicated that these observations are consistent with the idea that environmental effects are dominant in the early stages, and that time cumulative effects are stronger in the later stages of a lethal stress process. Shull et al.[99] considered a spore thermal death model that obeys first-order kinetics and that has a first-order dormant spore activation component. They described how this model may explain an initial lag period that is sometimes observed in semilogarithmic survivor curves.

Abraham et al.[1] carried out experimental survivor curve studies using *Bacillus stearothermophilus* spores in aqueous suspension at six constant temperatures ranging from 105°–130°C. The survivor curves displayed initial shoulders before log-linear declines. They used their data to test the idea of Shull et al.[99] mentioned above. Mathematical analysis showed that this model could represent not only their experimental survivor curves, but also all other shapes of survivor curves (linear and biphasic) found in the literature. Also, there was an inherent symmetry in the model formulation between the activation and destruction reactions. Abraham et al.[1] showed that the dormancy rate was the only parameter that permitted a distinction between the two reactions. The reaction rate values, K_D-values, were converted to D_T-values, which were plotted on a thermal resistance curve and were well represented by a straight line with a z-value of 8.6°C.

The D-value is one of the most useful tools in quantitative microbiology. It is important to understand and use it properly. In the most basic and accurate sense, the D-value is the cotangent of a line on a graph where data are plotted in the format $\log N$ on the y-axis versus U minutes on the x-axis. Experimental survivor curve data are plotted on a graph of $\log N$ versus stress time U. When the data can be represented by a straight line, a line is drawn through the data points, either freehand or using the parameters obtained from a linear regression analysis of $\log N$ versus U. To indicate the direction of the line, the cotangent instead of the tangent is calculated, and is assigned the symbol, D, for decimal reduction. The cotangent of the line (the D-value) is the time for the line to cross one logarithm. This is also the time for a 90% reduction in the numbers of surviving microorganisms.

Survivor curve data from most homogeneous populations of spores and microbial cultures, gathered under ideal conditions, form straight-line semilogarithmic survivor curves and can therefore be assigned D-values. The D-value reported will always be the D-value for the specific culture of microorganism tested using a specific methodology and recovery medium, etc. Neither the microbial species nor the strain has an intrinsic D-value. For a given species of microorganism, therefore, a range of D-values should be expected from the literature. For example,

B. *stearothermophilus* spores subjected to high temperature moist heat usually have $D_{121.1°C}$-values between 2 and 6 minutes; when *C. sporogenes* spores are tested under moist-heat conditions, they usually have $D_{121.1°C}$-values between 0.5 and 1.2 minutes.

See Casolari[27] for a more in-depth review of microbial death kinetics and models of microbial inactivation.

3.2.2 Graphical Presentation of Microbial Survival

Figure 3-1 shows a straight-line semi-logarithmic survivor curve. In practice, however, when the survivor curve data for apparently homogeneous microbial spore populations subjected to moist heat are plotted on semi-logarithmic graphs, the resulting curves are not always straight lines through N_0, as indicated in Section 3.2.1. Microbial survivor data do not always fit the logarithmic model exactly:

$$\log N = -U/D_T + \log N_0$$

The shape of the survivor curve varies with spore crop, substrate, and test temperature.[82] The most commonly observed survivor curves are shown in Figure 3-3. About 40% of survivor curves are approximate straight lines through N_0, like the curve labeled "a" in Figure 3-3. About 40% of survivor curves are of shapes "b," "c," or "d." The test results of some spore crops, substrates, and test temperatures are semi-logarithmic survivor curves that are sigmoid in shape (eg, "e" in Figure 3-3) or just curves (eg, "d" in Figure 3-3).

The caption to Figure 3-3 summarizes some of the explanations that may explain the various curve shapes. In addition, the medium used to estimate the number of surviving organisms after heat treatment can have a profound effect on the results. For example, the addition of starch, bicarbonate, and other supplements and of lysozyme to a medium can greatly increase the estimated number of spores of some *Clostridium* species that survive heat treatment.[59] The strength of the medium may also have an effect. In some cases, a more dilute medium may recover more heat-injured spores than a more concentrated medium.[121] In commercially prepared media, each batch is identified by a specific lot number and, because many natural ingredients vary, no two lots are the same and different lots from the same manufacturer may differ markedly in their ability to allow growth of heat-treated microorganisms. In a testing program to measure the effect of heat processes on microorganisms, it is essential to use the same media lot number in the evaluation of numbers of survivors in all tests where numbers of surviving microorganisms are to be compared.

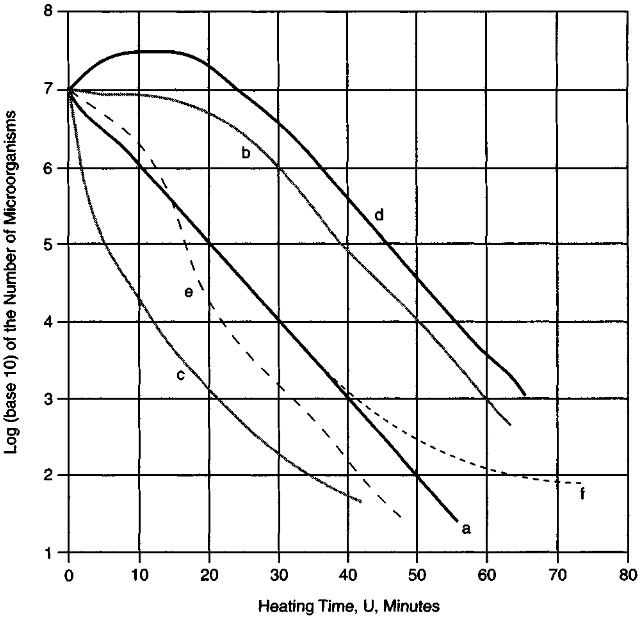

Figure 3-3 Microbial Survivor Curves. The primary shapes of microbial survivor curves (in the form log *N* as a function of heating time) obtained when bacterial spores are subjected to a heat process where the heating time is sufficiently long that heating and cooling errors are minimal: (a) straight-line classical, log linear, constitute about 40% of spore survivor curves, eg, *C. sporogenes* in phosphate buffer[82]; (b) concave downward curves are often described as having a shoulder; "multihit" inactivation processes have been proposed as a cause[64]; also, extensive clumping of cells can result in a delay before numbers of CFU begin to decline; 20% to 25% of survivor curves are this shape; (c) concave upward survivor curves result when there is a mixed microbial population[87]; also homogeneous cultures of microorganisms subjected to dry heat characteristically produce concave upward survivor curves[74]; 20% to 25% of survivor curves are this shape; (d) an initial rise in numbers of CFU prior to exponential decline may result from the need for significant heat activation of the spores before they are able to germinate; (e) combinations of all the above factors often result in sigmoidal-type curves such as the one with the dashed line; (f) classical curve (a) with a tail; tailing often occurs as a result of faulty experimental testing procedures or equipment,[77] or may be artifactual, resulting from the presence of small numbers of large clumps of cells in the population, or from heterogeneity, cell-to-cell differences of heat resistance within the population,[29,98] or even from "heat adaptation," an increase in resistance to heat occurring during the heating process.[50] *Source:* Copyright © 1989, G.W. Gould.

With respect to thermal processing, however, the fitting of a regression line to data forming curves like those illustrated by "d" or "e" in Figure 3-3 cannot be justified, other than to obtain a measure of the general direction of the data. Confidence limits should be indicated on numbers. Statistical aspects of the enumeration of microorganisms are reviewed in Chapter 60. Heat treatment can result in sublethal injury of microorganisms, which affects their ability to form colonies on recovery media (Chapter 15).

Deviations from true exponential survivor curves, similar to those sometimes observed with spores (Figure 3-3), occur to a greater degree and more often for vegetative cells.[3] This is particularly prevalent during lower temperature–longer time treatments when vegetative cells, unlike spores, may continue to metabolize and, thus, change in composition and structure, and consequently in thermal resistance, during the actual heating process. The so-called "heat shock" response (see below) can result in a substantial rise in microbial heat resistance, so that the resulting tails and shoulders on curves can be very pronounced and, again more so than with spores, can be greatly influenced by the heating menstruum and by the conditions of growth prior to heating. For example, Figure 3-4 illustrates survivor curves obtained with *Salmonella typhimurium* pretreated in various ways and heated in different menstrua. The variability illustrates well the difficulty in establishing unique thermal inactivation kinetics for vegetative cells, and the dangers of extrapolating one set of data to different environmental situations. However, it is generally maintained[116] that there is an underlying log:linear relationship on which these deviations are superimposed and, as with spores, similar explanations have been put forward to account for the variety of the shapes of vegetative cell survivor curves. These range from "single hit" models, as discussed above, to the assumption that the survival patterns predominantly reflect inherent phenotypic variations in the susceptibility of the individuals in a population to heat.[3] On this basis, heat is assumed to affect all cells in a completely uniform manner, and the shape of the survival curve is determined solely by nonuniform distribution of heat resistance within the population. Hansen and Reiman[51] assumed as much and concluded that the death of individual cells resulted eventually from accumulated damage.

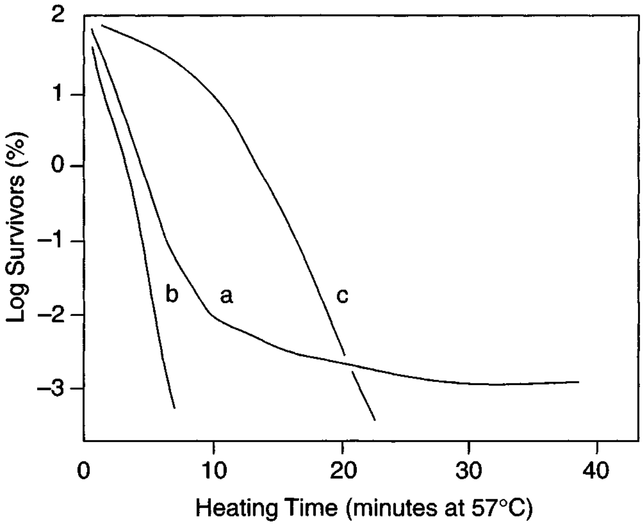

Figure 3-4 Heat Adaptation and the Influence of Heating Menstruum on the Heat Resistance of Vegetative Cells. Inactivation curves of *Salmonella typhimurium* heated at 57°C. Curve a: *S. typhimurium* Tm-1 grown in trypticase soy broth plus yeast extract (TSBY), washed, and heated in ground beef. From Thompson et al.[106] Curves b and c: *S. typhimurium* LT2, grown in TSBY and heated in the same medium without a prior heat shock (b) or following incubation at 48°C for 30 minutes (c).[61] *Source:* Copyright © 1989, G.W. Gould.

More recently, it has become well established that when vegetative cells of microorganisms are subjected to a variety of environmental stresses, including heat, they react homeostatically in such ways as to adapt to the stress. These adaptations involve the activation and expression of new groups of genes, eg, following irradiation,[65] osmotic shock,[17] oxidative stress,[37] starvation,[49,97] etc. The same is true for heat. Mild heating leads to the synthesis of "heat shock" proteins (eg, in *Escherichia coli,*[120] in *Neurospora crassa*[85]), in reactions that are widespread, occurring in all organisms that have been studied so far, including prokaryotes, eukaryotes, and humans.[95] Following synthesis of the new proteins, the heat resistance of cells rises. The rise may be transient, or resistance may remain elevated for long periods, depending on the conditions and on whether the synthesis of the heat shock proteins continues.

The extent to which the synthesis of heat shock proteins, and the consequent thermal adaptation, is important in food preservation and safety is not always obvious. Most of the practical uses of heat, in sterilization and pasteurization procedures, involve relatively rapid heat-up rates so that the opportunity for substantial thermal adaptation does not arise. However, when slow cooking procedures are used, eg, for large-volume cooked meats destined for chill storage and slicing at the point of retailing and for cooking in the home, thermal adaptation may be important in a limited number of cases. For example, Thompson et al.[106] showed that cooking large pieces of beef under conditions of constantly rising temperature could lead to a rise in the heat resistance of *S. typhimurium* from a $D_{50°C}$-value of about 30 minutes to a value of about 80 minutes during the cooking process. Mackey and Derrick[61] showed that the heat resistance of *S. typhimurium* at 50°–59°C could be increased several hundred-fold by prior incubation at 48°C for 30 minutes. When heated at steadily rising temperature, the greatest increase in resistance resulted when the temperature rose most slowly.[62]

3.2.3 Moist-Heat versus Dry-Heat Microbial Destruction

It has long been known that spores are killed quickly and at comparatively low temperatures in a steam autoclave compared with a dry-heat oven. Death rate is a function of water activity (a_w) or equilibrium relative humidity (ERH).[68] "Moist heat" is a condition where the a_w is close to 1.0, and all conditions below this are "dry heat," but heat resistance varies with a_w such that it is maximal at values near to 0.3 (30% ERH; Figure 3-5).

3.3 DETERMINATION OF RESISTANCE TO MOIST HEAT

It is not possible to instantly heat a sample of microorganisms, hold them at a test temperature, then instantly cool them prior to enumerating survivors. There will always be a heating and a cooling lag, depending on the volume of the substrate and the physical nature of the carrier. When short heating times are used, eg, less than about 3 minutes, the substrate volume must be very small, and the vehicle in which the heating is carried out designed in such a way that the sample will reach the test temperature in a fraction of a minute. The microorganism substrate carriers listed in Table 3-1 and the heating systems listed in Table 3-2 cover a range of conditions and types of heating medium.

3.3.1 Methods

A variety of laboratory methods have been developed for both *successive sampling* and for *multiple replicate unit sampling*. In general, successive sampling methods are used in survivor curve studies, while multiple replicate unit sampling methods are used for both survivor curve and "endpoint-quantal" studies (in which heated samples show growth or no growth). In successive sampling, the test suspension is in one single container that is sampled at intervals, so that the survivor curve reflects changes in the microbial population in one unit. In multiple replicate unit sampling, identical replicate test units are each sampled only once. Usually there are three to five replicates, enumerated in duplicate, for each of five or more heating times in a survivor curve experiment. In endpoint-quantal studies, 10 to 20 replicates at six or more heating times should be used when the results are to be scored on a growth/no growth basis.

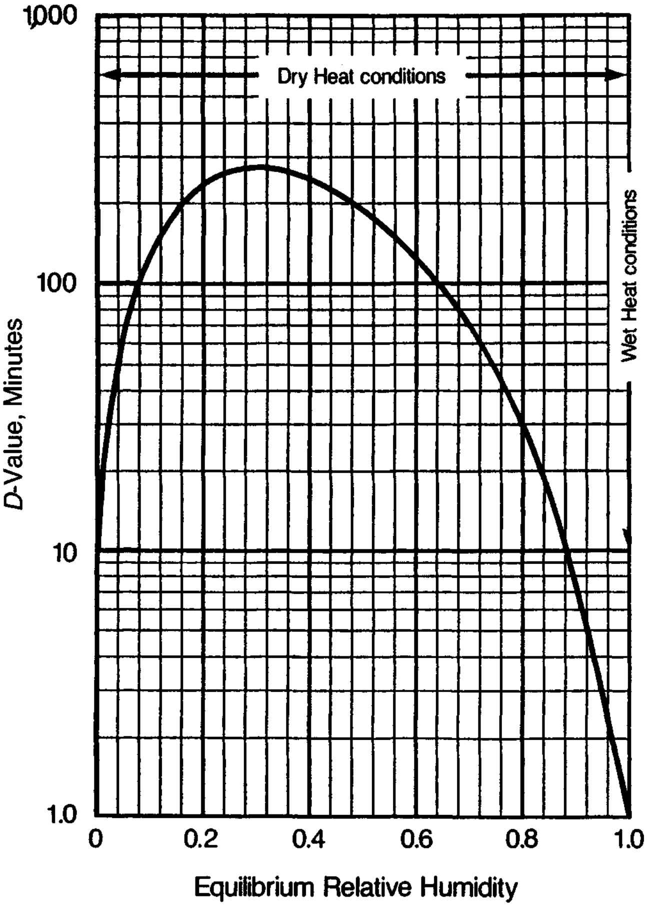

Figure 3-5 General Dependence of D_T-Values on Water Activity (a_w; scale 0 to 1.0) or Equilibrium Relative Humidity (ERH; scale 0 to 100%). *Source:* Copyright © 1995, I.J. Pflug.

Successive Sampling Systems

The primary apparatus is the flask or bottle containing the test substrate, which is submerged in a large water or oil bath with sensitive temperature control, and equilibrated at the required temperature. A small volume of suspension is inoculated into a large volume of heated diluent and samples

Table 3-1 Inoculum Substrate Containers Used in Multiple Replicate Unit Methods To Determine the Heat Resistance of Microorganisms

Container	*Substrate + Inoculum Volume (ml)*	*Heating Apparatus Required*	*Suitability of Method for Substrate Incubation or Culture*	*Heating Lag Correction; z = 10°C or 18°F (minutes)*
Glass tube, sealed	2	Waterbath to 90°C; above 90°C, oil bath, miniature autoclave or retort	Subculture or substrate incubation	0.9 to 1.2
Glass tube, 18 × 150 mm, screw capped	2–5	Waterbath to 90°C; above 90°C, oil bath, miniature autoclave or retort	Subculture or substrate incubation	2.0 minutes for 5 ml water in 18 × 150 mm test tubes (unless preheated)
Metal tube A1, 150 × 3 mm	1	Waterbath to 90°C; above 90°C, oil bath, miniature autoclave or retort	Subculture only	0.2 for 1 ml water
Capillary tube, glass	0,01	Special oil bath, cooling bath and transfer mechanism	Subculture only	Negligible
TDT can*	13 to 16	Waterbath to 90°C; above 90°C, oil bath, miniature autoclave or retort	Substrate incubation	0.6 to 2.0 in steam
Cup	0.01 to 0.02	Miniature autoclave or retort	Subculture only	Negligible
Cup-can system	0.01 to 0.02	Waterbath to 90°C; above 90°C, oil bath, miniature autoclave or retort	Subculture only	Negligible
Cup-resistometer	0.01 to 0.02	Thermoresistometer	Subculture only	Negligible

*Thermal death time cans: 208 ×006 (63.6 mm diameter ×9.52 mm deep).
Source: Copyright © 1995, I.J. Pflug.

are removed periodically. Unless these systems are completely closed and completely immersed in the heating bath, they suffer from the problem of surface cooling, which can lead to artifactual "tails" on survivor curves, particularly when the tests cover many log cycles of destruction.

Multiple Replicate Unit Test Systems

Sealed glass tubes. Suspensions of bacteria are filled into 7- to 9-mm OD Pyrex glass ampules; the filled units are heat sealed, then heated in a water bath, oil bath, or biological indicator-evaluation resistometer (BIER) system (described below). After the tubes have been heated they are scored, broken, and the contents removed. This was the procedure used by Bigelow and Esty[14] and is still widely used.[60]

Screw-capped tubes. Pyrex glass test tubes with screw-capped closures can be used in the same manner as heat-sealed glass tubes. They are reusable and eliminate the heat sealing and breaking steps; however, their heating lag corrections are large. A screw-capped tube technique is available that makes possible accurate heating at temperatures above 100°C.[55] Tubes containing the test liquid are preheated, submerged in an oil bath, then a small sample of spores or vegetative bacteria is injected into each tube, while still submerged, through a neoprene septum using a Hamilton syringe. At the end of heating, the tubes are cooled in an ice water bath. This method essentially eliminates heating lag corrections; however, there is a small test temperature drop when the spores or bacteria are added. The method can be used for both plate count and endpoint-quantal analyses.

Capillary tube method. This method[101,117] is similar to the sealed-glass tube method, except that the diameter of the tube is reduced to a capillary so both heating and cooling take place rapidly. Tubes are of the order of 1.7 to 1.9 mm OD with a 1.0 to 1.3 ID; volume is about 0.05 ml.

Metal tube method. Metal tubes,[63,70] with diameters between those of capillary tubes and 7- or 15-mm OD glass tubes, eg, 150 mm long, 3 mm in diameter, and volume 1.0 ml, are very useful test units when heating times need to be short. They have a

Table 3-2 Heating Equipment Used in Multiple Replicate Unit Testing Systems

Heating Equipment	*Units That Can Be Accommodated*	*Capacity of Units*	*Complexity of Operation*	*Utilities Required*	*Relative Cost*
Waterbath	Glass tubes, sealed, cotton plugged, screw capped; metal tubes; TDT cans*	Limited by physical size of apparatus	Simple	Low pressure steam or electricity	Low
Oil bath	Glass tubes, sealed; metal tubes; TDT cans	Limited by physical size of apparatus	Simple	Electricity	Low
Miniature steam autoclave or retort	Glass tubes, sealed, screw capped; metal tubes; TDT cans; cups, cups in cans	Limited by size of apparatus	Simple	80 psig steam	Moderate
Thermoresistometer	Cups	5 or 6 cups, depending on design	Complex	80 psig steam or electricity	Expensive

*Thermal death time cans.

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small lag correction factor (about 0.2 minutes) because the metal surrounding the column of substrate has a high heat conductivity and the column is small in diameter. The ends of the tube are internally threaded, and each end is closed with a plastic screw cap and O-ring. Metal tubes can be heated in an oil bath or a BIER vessel, and they are reusable.

Can method. Metal cans 2.5 inches (63.3 mm) in diameter by 0.375 inches (9.52 mm) high (in sanitary can terminology 208 × 006) were designed by the American Can Company[107] for use in thermal destruction studies of bacteria where the test product may be solid or contain large particles in a liquid.

Submerged coil method. In this procedure, a suspension of microorganisms is injected rapidly into a preheated, small-bore metal coil immersed and pre-equilibrated in a heated water bath so that heat-up time is short. An automatic syringe displaces aliquots to expel samples at predetermined intervals. The advantages of the method include the possibility of very rapid sampling rates.[33]

When heating times are very short, gathering accurate heat destruction data is problematic because of heating and cooling lags. An older method of treating heating and cooling lags is to calculate correction factors.[77] Another method is to calculate the heat effect during the thermal destruction experiment by accurately measuring the temperatures of the test sample during the heating up, holding, and cooling parts of the test, and summing the data, using the General Method Calculation Procedure.[54,77] For example, in a study of the effect of heat on spores of nonproteolytic *C. botulinum*, temperatures were recorded at 5- and 6-second intervals on a data logger and the information was analyzed on a microcomputer.[71] For each measurement temperature (T_m) the lethal rate (L) with reference to the target temperature (T_t) was calculated using the formula $L = {}_{10}[(T_m - T_t)/z]$ and a z-value of 8.0°C. The lethality of the treatment as equivalent time (minutes) at the target temperature was calculated from these data.

In determination of lags during heating and cooling, ie, the time required for the sample to reach the specified temperature, and in the determination of correction factors, it is essential that equipment for temperature measurement should be calibrated against a certified reference before and after each test.

3.3.2 Heating Systems

All heating systems must have sensitive temperature control and accurate temperature measurement (Table 3-2).

Waterbath. A waterbath is the ideal heating unit for temperatures below about 96°C. Baths should be equipped with efficient stirring devices to ensure uniform temperature and should be validated before use. The point-to-point or time cycle in temperature should be no more than 0.2°C. The lag correction factors for all types of product container units must be determined in the waterbath under the same conditions as will be used in the test. Units heated in a waterbath must be totally submerged during the time in the bath (see below).

Oil bath. An oil bath can be used for thermal inactivation testing over the range 95°–120°C. The upper limit is usually imposed by the heating lag in relation to the rate of inactivation of the spores, the lower limit by the more convenient use of a waterbath. The same type of temperature validation should be used as for a waterbath. The bath must be equipped with an effective stirrer. The heating lag correction is much larger when heating in oil than when heating in water or steam. Failure to totally submerge a test unit will usually result in a few survivors in the cooler out-of-bath part of the unit at time when the organisms in the bottom of the unit have all been killed, and therefore lead to tailing of log survivor curves.

BIER/steam vessel (miniature retort). The Association for the Advancement of Medical Instrumentation (AAMI) in Arlington, Virginia, developed standards for laboratory equipment to evaluate biological indicators used to validate and monitor sterilization processes. The equipment to mimic a steam autoclave was designated as a BIER/steam vessel (biological indicator-evaluation resistometer for saturated steam). This terminology is now often used to mean a small (miniature) saturated steam autoclave or retort where the test temperature can be accurately controlled. The BIER vessel[84,96] is a small pressure vessel that is capable of a rapid come-up-to-temperature. The retorts are usually connected, using large piping and quick action–type valves, to a large reservoir tank where the steam temperature is accurately controlled. The sequence of actions and temperature cycling in modern thermoresistometers has been further improved by computer control.[21]

3.4 SELECTING THE F_T-VALUE FOR LOW-ACID CANNED FOODS

The F_T-value is the sum of the lethal effects of a heat process expressed as minutes at a specified reference temperature, T (see definitions in Appendix 3-A), and forms the basis for establishing thermal processes.

When examining the available alternatives for systems to aid the establishment of microbial control values for low-acid thermally processed foods, the straight-line semi-logarithmic microbial destruction model is the accepted empirical approach to having an engineering model to use in the design and validation of microbial control processes. The equation that describes this model for moist-heat microbial destruction is

$$\log N_U = \log N_0 - U/D_T$$

where N_U = the number of microorganisms suviving a heat process of U minutes at temperature T; D_T is the decimal reduction time of the particular microorganism at temperature T, and N_0 is the initial microbial load.

Here is the equation rearranged in terms of U:

$$U = D_T (\log N_0 - \log N_U)$$

While it is appreciated that the semi-logarithmic model does not accurately fit all microbial destruction data,[80] to date we know of no other model that fits all experimental data. In practice, the appropriate parameters, N_0 and D_T, of the model can be adjusted so the model represents a worst-case condition (the greatest degree of safety) based on actual microbial destruction data. Fortunately, the endpoint term, N_U (numbers of survivors at the end of the process), of the semi-logarithmic model seems to have been designed to fit with the idea that the endpoint of a microbial control process should be a numerical specification. If the endpoint specification for spoilage by a mesophilic spore former is one nonsterile unit in 10^6 units, then on a unit basis, one out of 10^6 units is nonsterile, and the N_U-value will be $1/10^6$ or 10^{-6}. Therefore, the semilogarithmic model not only acts to relate the microbial destruction variables, but also aids in realistically describing the endpoint of the process, provided we can make some reasonable estimate of N_0 in practical situations.

There are then a few basic ideas in the approach to microbial control processes: (1) the experimental observations that one-celled organisms die in a near-geometric progression; (2) the selection of the F_T-value for canned foods should be approached in the order listed in Exhibit 3-1; and (3) process failure will usually be due to two or more independant changes in the process that act together. There are probably few one-cause failures.

3.4.1 Limitations in the Processing of Low-Acid Canned Foods

A canned food product may spoil either from growth of microorganisms surviving the heat process or from organisms that contaminate the product after it has received the heat process. With the

Exhibit 3-1 Priority Order for Design of Low-Acid Canned Food Process F_T-Value

1. ***Safe from a public health standpoint***[*]
   Probability of a viable *C. botulinum* spore surviving in a container of food should be of the order of 10^{-9} (PNSU-PH = 10^{-9}).[†]
2. ***Preserved against spoilage by nonpathogenic microorganisms***
   The term "commercial sterilization" has been widely used to cover this aspect of the microbial control process.
3. ***Preserved against mesophilic spore-forming organisms***
   Probability of a mesophilic organism surviving the microbial control process should be of the order of 10^{-6} (PNSU-M = 10^{-6}).[‡]
4. ***Preserved against thermophilic spore-forming organisms***
   Probability of a thermophilic organism surviving the microbial control process for a can of food to be stored below the growth range of thermophilic organisms (30°C) should be equal to or less than 10^{-2} (PNSU-T = 10^{-2}).[§] If the canned food is stored at elevated temperatures, the probability of survival should be 10^{-6} (PNSU-T = 10^{-6}).

[*] A minimum heat treatment sufficient to give a 12 decimal reduction in numbers of spores of *C. botulinum* is specified. If a maximum number of 1,000 spores of *C. botulinum* per container is assumed, this would give a probability of survival of approximately one spore in 10^9 containers.

[†] PNSU-PH: Probability of nonsterile unit-public health concern.

[‡] PNSU-M: Probability of nonsterile unit-mesophilic spoilage concern.

[§] PNSU-T: Probability of nonsterile unit-thermophilic spoilage concern.

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production of millions of containers of a food product per annum throughout the world, some will not be hermetically sealed, and it is possible for microorganisms to enter nonhermetic containers. However, the probability of occurrence is not the same for both failure modes, as discussed by Pflug and Odlaug.[82] Post–heat processing contamination is a problem that must be addressed and controlled quite separately from the destruction of contaminating microorganisms by the heat process.[90] The increased use in the 1990s of two-piece metal cans and three-piece metal cans with a welded side seam has greatly reduced post-processing contamination.

In the determination of the F_T-value for heat process design purposes, microorganisms are assumed to be at the slowest heating zone of the container of product, or to be uniformly distributed throughout the product. Of course, it is accepted that this is not necessarily so, and the implications of microbial location on the design of the heat process were discussed in detail by Ball and Olson[9] and Stumbo.[103]

3.4.2 Determination of the Heat Process F_T-Value

In the heat processing of low-acid canned foods, the first consideration is that the product should not be a public health hazard.[112] Spoilage that represents an economic loss is considered to be secondary to public health considerations. Economic loss may occur from spoilage either by mesophilic or by thermophilic spore-forming organisms (Exhibit 3-1).[113]

Selection of the heat process F_T-value ideally proceeds in a two-step fashion: (1) the F_T-value necessary for the product to be safe from a public health standpoint is calculated; (2) the F_T for preservation against spoilage by nonpathogenic mesophilic spore-forming organisms, and the F_T for preservation against nonpathogenic thermophilic spore-forming organisms are calculated. The larger value is then used to establish the retort or autoclave process. This approach is illustrated in Table 3-3 with the probability values used by Pflug.[77] Two generally accepted approaches to the selection of F_T-values are then:

1. ***The use of generally accepted empirical F_T-values.*** The experience of more than half a century of successful preservation of low-acid canned foods is available to the microbial control process design technologist in the form of empirical F_0 values (the F-values when T is 121.1°C (250°F) and the z-value is 10°C (18°F, see below). These have been proven over time, and hence have broad, safe applications. Procedures have been developed to take advantage of any antimicrobial-generating attributes of the food product.
2. ***The design of the microbial control process specifically for the product container unit.*** In this method, one uses an accepted model, where the D_T-value and, on the basis of available experimental determinations, an estimate is made of the maximum number of the critical microorganisms in or on a product. This allows for examination of the design of the preservation process in great detail, resulting in the development of a better understanding of the factors that impinge on the final F_T-value. The input values in this design will come primarily

Table 3-3 F_0-Values for Low-Acid Canned Foods

Hazard	*Endpoint Probability**	F_0 *(minutes)*†
Public health	10^{-9}	3‡
Preservation		
—Mesophilic spores	10^{-6}	3–7§
—Thermophilic spores		
Warehouse and distribution temperature below 30°C	10^{-2}	3–7§
Warehouse and distribution temperature above 30°C	10^{-6}	15–21§

*Endpoint probability of survival of organisms in a unit. This assumes a probable maximum initial number of 10^3, as well as the probability of survival (eg, 10^{-12} for spores of proteolytic strains of *C. botulinum*).

†F_0 is the equivalent minutes at 250.0°F (121.1°C) calculated using a *z*-value of 18.0°F (10.0°C).

‡Based on the laboratory data of Esty and Meyer.[42]

§Based on industry experience (empirical data).

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from experience, but should be adjusted for local conditions.

Calculating F_T-Values Using the Straight-Line Semi-Logarithmic Model

A case has been made previously for using the straight-line semi-logarithmic model as the heat process (F_T-value) design model. In the following development, the model is used on a container basis. In using the model,

$$F_T = D_T(\log N_0 - \log N_F)$$

F_T = heat process value, equivalent minutes at temperature T, z-value taken as 10°C (18°F)

D_T = decimal reduction time, time in minutes for a 90% reduction in the critical resistant microbial population

N_0 = initial number of critical resistant microorganisms per unit

N_F = the endpoint of the preservation process, the number of surviving microorganisms per unit

The endpoint value (N_F) is a consumer protection value, and is hence not product specific. The values N_0 and D_T are product specific.

F_0-Values for Low-Acid Canned Foods

Table 3-3 shows practical F_0-values for low-acid canned foods. These are the commonly accepted best estimates of the net F_0 required for microbial control. Data to support the "empirical F_0-values" in Table 3-3 are from different sources. This is possible because of the nature of industry experience with low-acid canned foods, and the F_0-values necessary for preservation against spoilage by nonpathogenic mesophilic and thermophilic spore formers. We do not have an empirical database showing survival of *C. botulinum* spores in cans. Consequently, we must use laboratory data as the basis for the public health, microbial control value against *C. botulinum.*

The empirical F_0-values of 3 to 7 minutes given in Table 3-3 for mesophilic spores are validated by industry practice of using a 5-log reduction of *Clostridium sporogenes* PA3679 spores having a $D_{121.1°C}$-value of 1.0 to 1.5 minutes as an adequate design. We estimate the practical $D_{121.1°C}$-value for resistant mesophilic spores in foods to be about 0.5 to 0.8 minutes, calculated using the straight-line semi-logarithmic model with an N_0 of 10^1 to 10^4 per unit and an endpoint value PNSU of 10^{-6}. The apparent resistance of the resistant mesophilic spores in the product is therefore approximately half of the maximum resistance value.

Safety from a Public Health Standpoint

The accepted approach to *C. botulinum* control is to use the F_0-value of Esty and Meyer[42] as corrected for heating and cooling stages by Townsend.[107] When this number (2.45 minutes) is rounded up, it becomes the F_0 of the 3.0 minutes *C. botulinum* process shown in Table 3-3.

The number of *C. botulinum* spores per container will vary with the food. For most low-acid foods, the initial number per container may be of the order of 10. For mushrooms, the N_0 may be as high as 10,000. For some meat products, the N_0 may only be one or fewer spores in 100 containers (<0.01 per container). For packaging materials and also for preformed packages used in aseptic packaging systems, there may be only one *C. botulinum* spore in or on the material to produce 10^5 packages; that is, 10^{-5} organisms per package.

Regarding the F_0-value necessary to ensure safety, Esty and Meyer[42] used approximately 10^{11} of the most resistant *C. botulinum* spores that they could grow per unit tested, and used as their endpoint no growth in about 10 replicates, which was a survival level of no more than 10^{-1}, so the log reduction for their experiments was about 12. The F_T-value developed by Esty and Meyer therefore will reduce a practical upper level of 10^3 *C. botulinum* spores per container to an endpoint probability of 10^{-9}. Today, more than 75 years after publication, these data are still quoted as the basis for selecting the minimum

F_0-value for preserving low-acid foods against botulism. The work of Esty and Meyer[42] is still one of the few published papers that provides data for validating an 11- or 12-log destruction of *C. botulinum* spores.[76]

Of course, the $D_{121.1°C}$ of 0.2 minutes does not necessarily represent the resistance of *C. botulinum* spores in food products, but tends to be the *maximum* value that could be produced in the laboratory. The *C. botulinum* spores that contaminate food raw materials are formed in nature. Because it is known that the growth medium affects spore resistance, it is probable that naturally occurring spores have different resistances from those determined in the laboratory studies. In this sense, the figures used so successfully for many years are probably conservative, and considerably "fail-safe"; on the other hand, the constituents of some foods are known to protect spores from heat inactivation.

In relation to the safety of meat products, extensive efforts have been made to ensure that meat does not contain the prions that are thought to cause bovine spongiform encephalopathy (BSE) and "new variant" Creutzfeldt-Jakob Disease (v-CJD; Chapter 57). The limited data available regarding the heat resistance of these prions indicate a heat resistance similar to that of the agent of scrapie,[105] which showed nonexponential inactivation kinetics.[22] Casolari[28] used these and other data to conclude that current thermal processes applied to foods would deliver little inactivation of these prions.

3.4.3 Preservation against Spoilage by Nonpathogenic Thermophilic Spores

The ambient temperature conditions of warehouses and distribution systems in some parts of the world are such that thermally processed foods that may be perfectly stable in cooler climates may run the risk of spoilage by thermophilic spore formers. Since many of these have heat resistances well above those of mesophilic spore formers and *C. botulinum*, especially severe heat processes may be necessary for their control. Thermophilic spore formers that spoil low-acid canned foods are often divided into three groups: (1) those that produce flat-sour spoilage (eg, *Bacillus stearothermophilus* and *B. coagulans*); (2) those that produce gas, but not hydrogen sulfide (eg, the thermophilic anaerobe *C. thermosaccharolyticum*; and (3) the thermophilic anaerobes that produce hydrogen sulfide spoilage (eg, *Desulfotomaculum nigrificans*, formerly *C. nigrificans*).

B. stearothermophilus and *B. coagulans* are the chief flat-sour thermophiles found in canned foods.[10,52,116] While both cause flat-sour spoilage, they have different characteristics. *B. coagulans* is a problem particularly in canned foods exposed to temperatures in the range from 40° to about 60°C and at pH values of 4.3 to 4.8. The $D_{121.1°C}$-values for laboratory-grown spores vary from about 0.2 to 1.0 minute depending on the strain and substrate and on the medium in which the spores were grown, heated, and recovered. *B. coagulans* is not a major flat-sour spoilage organism of low-acid canned foods, and its heat resistance is not much greater than that of the mesophilic spore formers discussed previously.

B. stearothermophilus is the primary cause of thermophilic flat-sour spoilage in low-acid canned foods because it will grow in the pH range from 5.4 to above 7, and has a $D_{121.1°C}$-value that is 5 to 10 times that of the resistant, mesophilic spore-forming organisms. *B. stearothermophilus*–type organisms sometimes may grow at temperatures as low as 37°C, but never below 30°C.[10,116]

C. thermosaccharolyticum is a very heat-resistant saccharolytic, thermophilic anaerobe that produces gas, and hence swelled-can spoilage. The optimum growth temperature is about 55°C; only rarely has growth been reported at temperatures as low as 30°C. Strains of this bacterium cause significant spoilage, especially in the pH range from 4.5 to 5. They are very heat resistant; *D*-values for spores at 121.1°C were reported as 3 to 4 minutes[103] and for one strain, more than 50 minutes.[119] It is usually not possible to produce an acceptable canned food product if the heat process must be designed to kill large numbers of *C. thermosaccharolyticum* spores. Canners try, therefore, to test batches of ingredients for this bacterium and to select for use batches with the minimum numbers of this organism.

Organisms of the group *Desulfotomaculum*, heat-resistant thermophilic anaerobes that produce hydrogen sulfide, are responsible for the so-called "sulfur-stinker" spoilage of canned foods. These organisms are only feebly saccharolytic; cans with spoiled product usually remain flat, since the hydrogen sulfide is soluble in the product. The product becomes black as a result of the interaction between the sulfur compounds and the iron in the containers. *D. nigrificans* is one of the most heat-resistant thermophilic organisms. The temperature range for growth is usually 45°–70°C, with an optimum at about 55°C; the bacterium can be adapted to grow slowly at 30°–37°C. Using a plate count survivor method, $D_{121.1^\circ C}$-values for spores of strain ATCC 7946 of 3.2 to 4.0 minutes in water and phosphate buffers, and 5.6 to 7.6 minutes in various broths have been reported.[39] When fraction-negative tests were used, a $D_{121.1^\circ C}$-value of 25.8 minutes was obtained for isolate ATCC 7946 and 54.4 minutes for isolate RGI.

In spite of these enormous resistance values, spoilage of foods by *D. nigrificans* tends to be rare. This may be due in part to the fact that because of the severe heat processes required, it may not be possible to produce a canned food of acceptable quality when a product contains large numbers of *D. nigrificans* spores; hence, only raw materials that have very low N_0 levels of these organisms are used.

If temperatures are expected to rise above 30°C during the expected distribution and/or shelf life of a canned food product, raw material quality and processing should be designed so that the probability of thermophilic spoilage should not exceed 10^{-6} for all product storage and handling conditions.

3.5 HEAT DELIVERY

There is a very wide range of methods available for the delivery of heat to foods. Some of them, eg, domestic cooking, are important from the standpoint of public health, but are not under strict control. On the other hand, the various methods of commercial processes for delivering heat to foods are tightly controlled.

3.5.1 Domestic and Commercial Cooking

One of the most important applications of heat is during food preparation to ensure the elimination of microorganisms including pathogens such as *Listeria monocytogenes*, *Salmonella* species, *Campylobacter* species, and pathogenic strains of *Escherichia coli*. The food cooking process is a most important step in making food microbiologically safe. When, in the cooking process, the slowest heating zone of a product is at or above 180°F (82.2°C) as is the case for most vegetable products, vegetative microorganisms and viral and fungal pathogens will be killed. Most vegetables are cooked by boiling. The end-of-cooking temperature for most vegetables is about 180°F, and starchy foods such as rice and potatoes are usually cooked to a temperature of 190°F (87.8°C) and above.

For chicken, pork, and game meat, the general recommendation is to cook to a temperature that will render the color of blood gray or brown, corresponding to a cooking temperature above about 165°F (74°C). If there are cooking processes and products that have special food safety aspects, these are with beef and lamb, where these products are often eaten in a rare or medium-done state. The USDA requirement for roast beef gives an indication of the microbial control capacity of the cooking operation. For salmonellae, they recommend a reduction in the initial load of 1×10^7 to one organism per gram of beef. (For ground beef, a suggested salmonella reduction is 5 log cycles.) A process of 121 minutes at 130°F (54.4°C), 12 minutes at 140°C (60.0°C), 1.2 minutes at 150°F (65.6°C), or 0.12 minutes at 160°F (71.1°C) is recommended for control.[109,111] These times and temperatures can be related to the American Meat Institute specifications for "doneness": 130°F produces very rare beef, 150°F produces medium beef, and 160°F produces well-done beef.

In baking, products are heated until the interior is at approximately 212°C (100°C), when the crust will be at a temperature slightly above the boiling point of water and steam will have been generated in the center of the product.

Simmering will result in a product temperature very close to the boiling point of water. The interior of products will generally reach at least 194°F (90°C). Specifications for food service and/or catering are to either keep food hot (above 140°F/60°C, except for beef: 130°F/54.4°C), or to chill it in less than 15 hours from 130°F (54.4°C) to 45°F (7.2°C) so that any surviving spores, especially of *Clostridium perfringens*, will not germinate and multiply.

In broiling and grilling, the core temperature will usually be of the order of 150°F (65.6°C) to 190°F (87.8°C), with the surfaces receiving radiant energy being at a higher temperature.

During frying, fat is the medium carrying heat to the product. For shallow pan- or deep fat-frying, the core temperature at the end of the frying period is commonly between 150°F (65.6°C) and 190°F (90°C). Similar temperatures will be reached during griddle cooking, which involves heating food on a hot metal surface without added fat.

In microwave cooking, heat is delivered predominantly via the excitation of water molecules in foods. The inactivation of microorganisms is determined by the temperature reached, just as with any other form of cooking.

3.5.2 Thermal Processing for Commercial Sterility

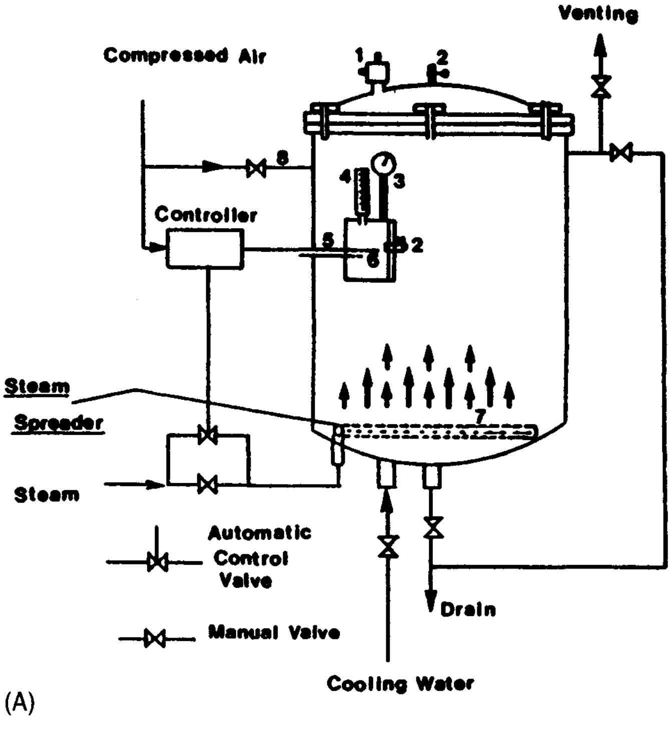
Still Retorts

The still (nonagitating, noncontinuous) retort, or autoclave, has been in use for more than 100 years as a device for processing low-acid foods above the boil-

Next Page

ing temperature of water. In many canning operations, the still retort has been largely replaced by continuous agitating retorts.[54,90] However, for the sterilization of conduction heating food products, for processing glass packed products, and for small companies that cannot justify the cost of an agitating retort system, the still retort remains the primary piece of heat processing equipment. Still retorts may be of a horizontal design, with the door at one end, or vertical, with the door at the top (Figure 3-6). Processing food in a still retort is a batch operation, however, the Malo system is unique in that the retort is filled and unloaded automatically. It is a crateless system where the cans are in a scrambled condition and can be handled as a fluid. However from the processing standpoint, it is a still retort.

General considerations that apply to the processing of containers in still retorts include the following: (1) In thermal process design, as part of the validation testing program, the slowest heating containers in a retort are identified and the process design is based on having an adequate process for these containers. (2) Heating rate data from heat penetration tests carried out in laboratory retorts and simulators can be used appropriately in the design of processes for commercial equipment. This is true in general but not universally; consequently, it is the responsibility of the sterilization technologist to determine the applicability of laboratory results and/or simulator results to the commercial equipment. (3) In general, the more complex the process, the more important it is that the test equipment be validated to ensure that the results from the test equipment are valid in the commercial equipment.



Heating media do not all perform in the same way, and can be considered in two classes: (1) 100% saturated steam and (2) water (with steam-air overpressure), water-spray (steam-air atmosphere), and steam-air mixtures. In a properly vented 100% steam retort, we have a saturated vapor heating medium in which the temperature is a function of pressure and, for practical purposes, the temperature at all container surfaces in the retort will be at the saturation temperature. All of the second class group of media require some type of mechanical mixing to keep the heating medium temperature approximately constant throughout the retort. In water and water-spray systems, pumps are used and in steam-air systems, large fans or blowers are used to circulate the heating medium.

Still retorts may be operated manually with very simple instrumentation or automatically with time/

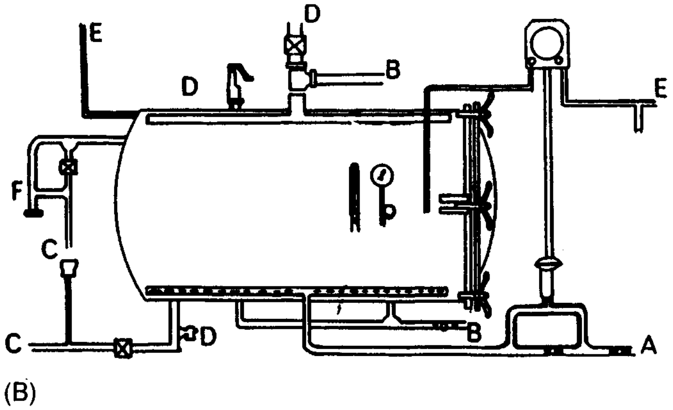


Figure 3-6 Batch Retorts. (**A**) A vertical batch retort: 1, safety valve; 2, petcocks to maintain a steam bleed during processing; 3, pressure gauge; 4, thermometer; 5, sensing element for controller; 6, thermo-box; 7, steam spreader; 8, air inlet for pressure cooling. (**B**) A horizontal retort: A, steam; B, water; C, drain, overflow; D, vents, bleeder; E, air; F, safety valves and pressure relief valves. *Source:* Reprinted from P.S. Richardson and J.D. Selman, Heat Preserving Equipment, in *Processing and Packaging of Heat Preserved Foods,* J.A.G. Rees and J. Bettison, eds., pp. 50–71, © 1991, Aspen Publishers, Inc.

temperature cycle controls. The expenditure that can be justified for instrumentation generally must be related to the complexity of the process. For example, steam cooking of small-sized metal containers is a rather simple operation and warrants only temperature control, whereas processing glass packed products under water with superimposed air pressure will require pressure, temperature, and time cycle control to achieve the same efficiency. When pressure controls are used, corrections must be made for altitude.

In the processing of medium-sized cans of foods in still retorts, where steam is used as the heating medium and the cans are cooled with water either in the retort or in a cooling canal, it may be necessary to pressure cool the cans so that their ends remain concave during cooling. Steam may be used to maintain the pressure if the cold water is introduced from the bottom of the retort under a layer of hot water. A better solution is to use compressed air. Products packed in glass containers must be processed under water with superimposed air pressure. Glass packed products are cooled in the retort under water until the temperature of the food product has been reduced to below 150°F (65.6°C). The remainder of the cooling may take place in a cooling canal. The temperature of canned foods should be below 100°F (37.8°C) when cans are placed in the warehouse.

Flexible packages containing low-acid food products must be restrained in a rack to control pouch thickness during the sterilization process. The racks must be designed so that there is space between pouches for the heat transfer medium. Over-pressure must be used during cooling to avoid pack rupture.

Agitating, Discontinuous, Pressure Processing Machines

In general, processing machines in this category have some type of basket into which the containers of product are loaded. During the heat process the basket rotates (Figure 3-7), agitating the contents of the containers in the basket, which increases the rate of heat transfer, shortens the process, and reduces the overcooking of product in contact with the container walls.

Early in the 20th century, Fort Wayne and Berlin Chapman sterilizers were developed and widely used for sterilizing evaporated milk. These retorts contained a removable, large, rectangular perforated basket into which the cans were stacked. The filled basket was moved into the pressure vessel. Driven by an externally located motor, the basket filled with containers revolved on its own axis during processing. The rate of basket rotation was of the order of 6 to 12 rpm. Starting in about 1950, for high-volume products, continuous agitating pressure sterilizers tended to replace the end-over-end batch machines. Continuous processing machines are economical in operation but tend to be large, expensive, and designed for one product in one container size. The intermittent axial rotation of the continuous spiral cooker is adequate for liquids such as milk, but is inadequate to process some of the newer more exotic products where there are particulates in thick sauces. End-over-end rotation offers more flexibility when it comes to difficult-to-heat items in glass containers, flexible packages such as the "retort pouch," and semi-rigid containers. In the 1960s, a new generation of rotating basket machines appeared to meet these needs. The heat transfer media are either pressurized water (Stock Rotomat, Klinge, Allpax, Hisaka) or an air-steam mixture (LaGarde, Steristar).

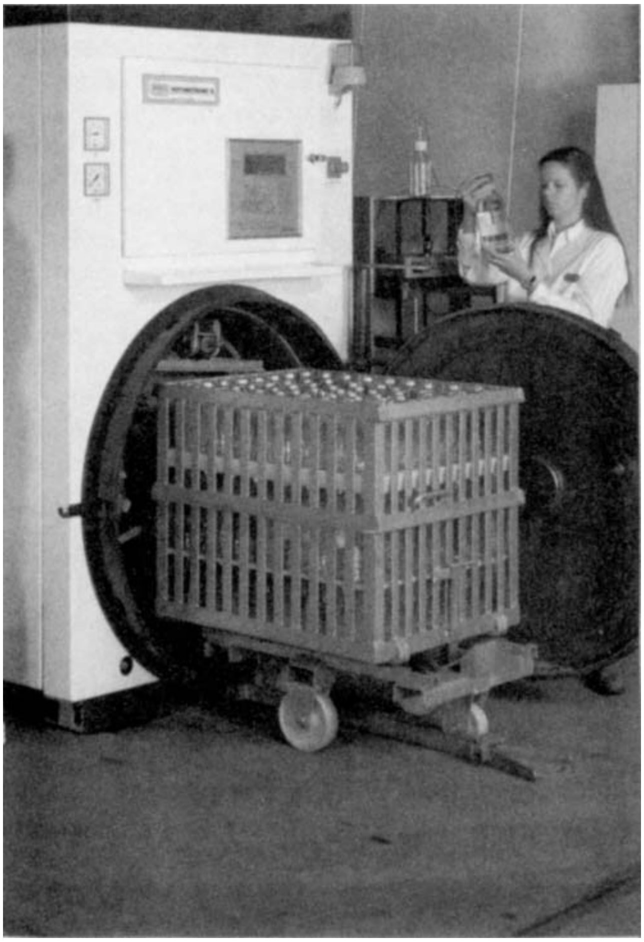


Figure 3-7 Rotating "End-Over-End" Sterilizer. Courtesy of STOCK America, Milwaukee, Wisconsin.

In general, today's rotary basket sterilizers have baskets or carts that are clamped into a rotating frame, and are part of a system where the individual baskets are at least semi-automatically loaded and unloaded. They are capable of handling a wide range of container geometries. The size and shape of the container, the position of the container in the container rack system, and basket rotational speed (20 to 30 rpm are used with viscous products) are selected so both the heat transfer and product quality needs are met. The slowest heating containers are at the axis of rotation. Those most distant from the axis of rotation receive the maximum agitation and consequently are likely to heat fastest. All present-day rotating basket machines utilize some positive means of ensuring uniformity of temperature of the heat transfer media through the crates. These processing machines are equipped with microprocessors and other types of computerized monitoring and control systems. Eisner[41] gives a full description of the operation and use of rotary basket sterilizers.

Continuous Hydrostatic Pressure Cooker–Cooler Systems

These systems are unique in being open so far as conveying is concerned, there are no valves or locks. A single chain conveyor with can-carrying flights is continuous throughout the entire system (Figure 3-8). Steam pressures of 10, 15, or 20 psi, necessary for sterilizing at 240°F (115.5°C), 250°F (121°C), or 260°F (126.7°C), are balanced by hydrostatic or water head pressures in the heating and cooling water legs. Net water heads of 23, 34.5, and 46 feet, respectively, are necessary to produce pressures of 10, 15, and 20 psi. The total tower height therefore varies with the temperature design; 40 feet for 240°F, 50.5 feet for 250°F, and 61 feet for 260°F. A minimum amount of floor space is required. A unit with a capacity of over 1,000 cans per minute occupies an area of only 13 × 26.5 feet.

Hydrostatic-type cookers are usually designed so that several can sizes can be used without major

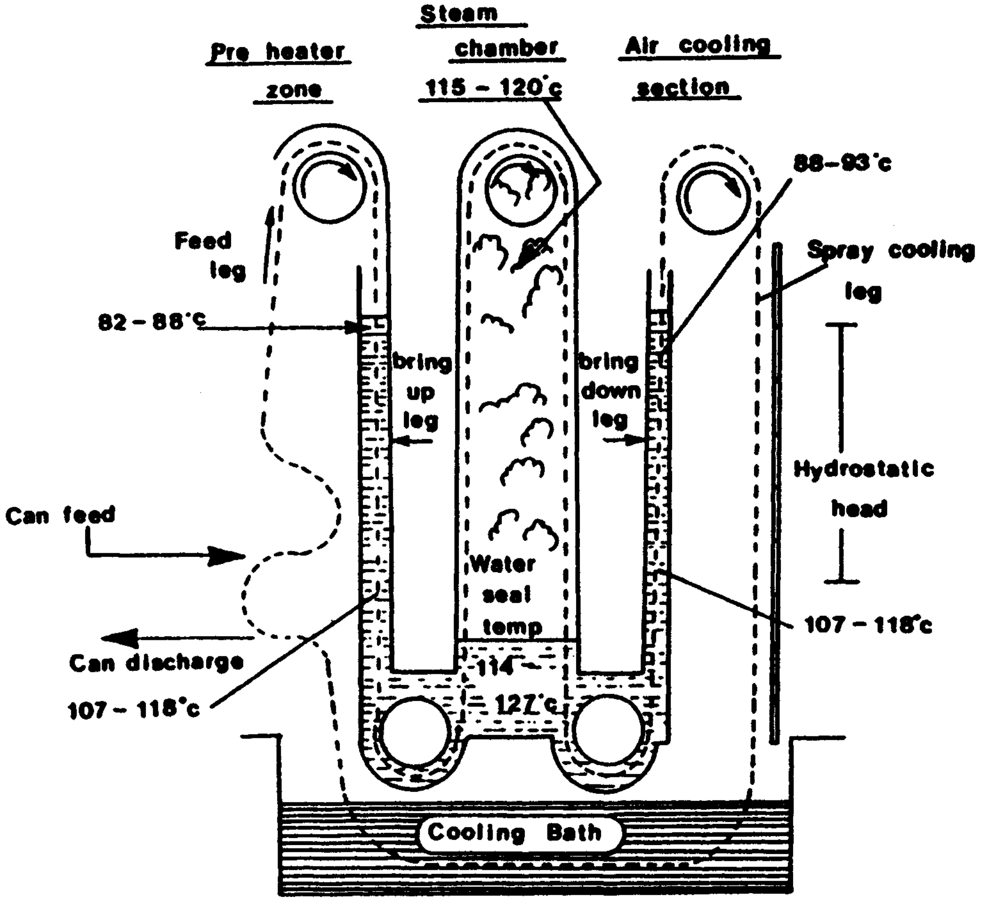


Figure 3-8 Hydrostatic Retort System. Courtesy of Food Machinery and Chemical Corp.

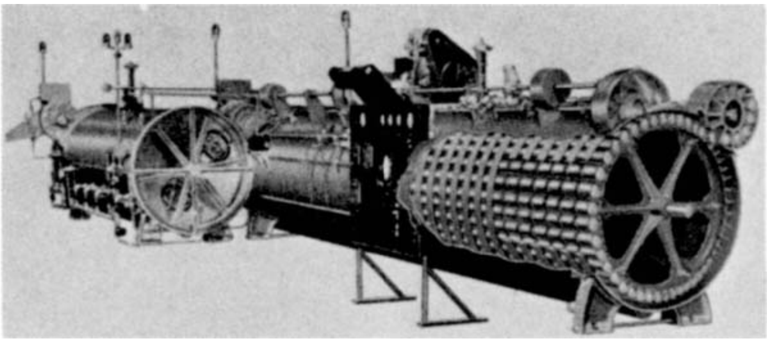


Figure 3-9 Cutaway View of a Continuous Agitating Pressure Sterilizer ("spiral cooker"; Food Machinery and Chemical Corp). *Source:* Copyright © 1995, I.J. Pflug.

equipment changes. In principle, the hydrostatic-type cooker is the least complicated continuous pressure cooker in use today. However, to make this simple principle into a practical piece of food processing equipment requires considerable mechanical equipment in the way of feeders, unloaders, conveyors, and controls. Corrosion is a constant problem. Efficiency is illustrated by a 3-in-1 hydrostatic sterilizing operation[93] that replaced 33 retorts and processed 35,000 cans per hour with savings of steam, water, labor, and floor space.

Continuous Agitating Pressure Sterilizers

The continuous agitating pressure sterilizing system (FMC) consists of separate units or components that are put together to form a sterilizing system (Figure 3-9). Components that are available are: preheater, pressure cooker, micro-cooler, pressure cooler, and atmospheric cooler. In general, the system is tailor made for a particular processing operation. In systems for sterilizing vacuum packed foods, it is necessary to have a preheater prior to the pressure cooker and a pressure cooler before the atmospheric cooler. The purpose of the preheating section, maintained at a moderate temperature and pressure, is to heat product to a temperature of 180°F (82.2°C) to 210°F (99°C), so that the internal pressure in the can is approximately one atmosphere (zero gauge pressure) when the cans enter the pressure cooking system.

The preheating, pressure cooking, and cooling sections all operate in the same manner. Each section consists of a cylindrical pressure tank inside of which is located a reel containing a helical track. The containers of food enter through a valve; they roll, slide, and are carried around until they reach the end of the unit where they are valved out. The containers receive a unique rotational pattern as they proceed through the units so that in a single revolution of the reel, the cans are carried around the top portion of the cooker, slide a short distance along the side, and then roll along the lower portion of the unit. The agitation received by the containers of food is sufficient to cause products that normally are considered to heat by conduction, such as cream-style corn, to heat almost as fast as normal convection heating products. Steam is used as the heating medium and cold water is used as the cooling medium. The units are equipped with automatic temperature controls in the heating section and water level controls in the cooling section, and conveyors are used to make a continuous processing system.

The advantages of the continuous agitating pressure sterilizing systems are that they reduce processing times and costs, and a uniform cook is obtained. High operating temperatures, 260°F (126.7°C) or 280°F (137.8°C), plus agitation substantially reduce the processing time for convection heating foods. Certain food products that are normally heated by conduction can be induced to heat by convection, resulting in a major saving in time and improvement in quality. Prior to 1950, continuous cookers were restricted primarily to milk, peas in brine, and tomato products. However, since 1950, with the development of a new type of cream-style corn, they have been widely adapted for the processing of this product as well as brine-packed corn, soups, and formulated infant foods.

Hydrolock System

The Hydrolock is the name given to a continuous agitating pressure sterilizing machine in which the containers move through the system in carriers on a chain similar to the handling system of a hydrostatic processing machine. The chain with the carriers makes several passes horizontally the long way of the cylindrical pressure chamber. The top section of the pressure chamber is filled with steam plus air. Cold water for cooling is in the bottom of the horizontal cylinder. The heating and cooling sections are separated by a baffle. Fans are used to circulate the heating medium when steam-air mixtures are used. The product carrier chains move in and out of the pressure chamber through a single rotary valve that is water sealed, hence the name Hydrolock. Containers may be continuously rotated by contact between the carriers and a belt directly below the carriers.

Hydrolock processing machines are used in Europe and Asia for commercial sterilization of fruits, vegetables, and milk products, and in North America for sterilizing pharmaceutical products. The Hydrolock machines can process products in both metal and plastic containers.

Flame Heating

Flame sterilization is a method of rapidly heating cans of food to effect sterilization by placing the cans in direct contact with a gas burner flame while they are rapidly rotated. The rotation induces convection heat transfer of the product in the container. The heat transfer rate from flame to can is very high because of the huge temperature difference even though the heat transfer coefficient is relatively low. Flame sterilization is recommended by the inventors for liquid food products and for particulate products packed in brine, syrup, or juice. The concept was developed by Cheftel in France.[13,32] Much work has been done on this process by Casimir in Australia[23–25] and Leonard in California.[56–58]

The chief attraction of the method is the rapid heating of the containers of product. The processing machines are continuous and have relatively low initial cost but relatively high energy consumption. The temperature of the gas flame varies between 2,000° and 2,500°F (1,090° and 1,370°C). The system operates at atmospheric pressure, therefore the can must withstand the internal pressure that is developed; a 3.2-inch (81-mm) can is probably the largest that can be processed satisfactorily. In general, the can end must be stronger (heavier plate) when flame sterilization is used than when the product is to be retort sterilized.

The most difficult problem of flame heating is that of obtaining uniform product temperature so that a constant *F*-value is delivered to the product in all the containers. Directing a flame onto a can and surrounding a can with hot gases is not as controlled a procedure as placing a can in a retort filled with steam. Therefore, there is a problem of underprocessing, eg, if a can misses the flame, the flame is not at the right intensity, or some other condition exists that results in inadequate flame contact time with the can.

The flame sterilizer is under a unique constraint. When product temperatures within the can exceed perhaps 280°F (138°C), the internal pressure will cause the can to fail with explosive violence. On the other hand, deflection of the flame for even a fraction of the short heating period will mean that very little heat is transferred to the container, and gross underprocessing will result. A system therefore has been developed whereby the temperature of each can passing through the system is monitored to ensure that all cans receive an adequate sterilization *F*-value. The limited control of the flame heating procedure will necessitate some such type of positive monitoring of all containers if this system is to be commercially acceptable for the processing of low-acid foods.[23–25]

3.5.3 Processing of Acid Foods

In commercial processing of foods in most countries, products with a pH of 4.6 or lower (US) or lower than 4.5 (UK) are deemed to be acid foods and can, in general, be made shelf stable by processing at temperatures below 212°F (100°C). Products such as canned acidified cucumbers and other vegetables, fruit juices, and tomato products are made shelf stable by a heat treatment in one of the following four types of equipment: waterbath heating systems, steam-air heating systems, water-spray systems, and continuous agitating atmospheric cookers.

Waterbath Pasteurizers

A waterbath is an ideal piece of equipment for the pasteurization of products such as pickles. Temperature control can be very good, the heating rate is comparable to that in water-spray or steam-air pasteurizers, and the equipment is simple to operate and relatively inexpensive. Many systems are being used, which vary according to the materials used for construction of the waterbath, the method of handling jars or other containers, and the procedure used in heating and cooling. Wooden tanks, vertical type three-crate retorts (with the lids removed) and fabricated steel tanks are all used. The methods of handling the jars or containers vary. In some cases, the jars are hand stacked into wooden tanks, the tanks are filled with water, and the water heated by the direct injection of steam. This is one of the original methods of making fresh cucumber pickles, though not used extensively in large operations. Retort baskets or crates for holding jars or cans of pickles and three-crate vertical retorts are still used in many maufacturing plants. In others, rectangular tanks with fabricated rectangular baskets are used. Where baskets or racks are used for holding the pickle jars, the procedure is to place the containers of pickles in water that has been preheated to 120°–140°F (49°–60°C). During the heating portion of the cycle, the water is maintained at 170°, 175°, or 180°F (77°, 79°, or 82°C). To cool the pickles, cold water can be added to displace the hot water. Otherwise, baskets of product are moved sequentially from preheating tanks to pasteurizing tanks, then into tempering tanks and into cooling tanks.

The waterbath system is, for all intents and purposes, a batch system, and has the disadvantage of high labor requirements. It is best adapted to small or specialty operations where the quantity of product does not warrant the installation of a continuous system.

Steam-Air Pasteurizers

The typical steam-air pasteurizer is a rectangular tunnel 7 feet wide, 2 feet high, and about 100 feet long and open at both ends. An endless-type stainless steel chain conveyor passes through the tunnel and conveys the product continuously through the unit. The pasteurizer is usually constructed in 10-feet long sections with canvas baffles between the sections to keep air currents from blowing the steam-air mixture out of the tunnel, and consists of a heating area, holding area, and cooling area. The heating sections are fitted with steam pipes containing small orifices; the steam pipes are usually located at the top of the pasteurizer. Cooling is carried out by water sprays. The cooling section is equally as critical as the heating section, and high cooling water temperatures can limit production rates. The jars of product, such as pickles, enter the pasteurizer and proceed through one section after another, where heat flows into the containers. They are held in a nonheating, noncooling section for a few minutes for tempering, then moved into the cooling section where they are cooled to warehousing temperature.

The advantage of the steam-air pasteurizer is that it is a continuous processing unit; corrosion in the heating section is reduced over water-spray–type pasteurizers through a reduction in the quantity of equipment that is used and that comes in contact with corrosive fluids. The disadvantages are principally those associated with temperature control of the pasteurizer.

Water-Spray Pasteurizers

The water-spray pasteurizer is very similar to the steam-air pasteurizer. The difference is that whereas the steam-air pasteurizer uses a gaseous steam-air atmosphere as a heating medium, the water-spray pasteurizer sprays heated water over the containers. The cooling section is the same for both types of systems. Pflug and Nicholas[81] found that water sprays were very effective in heating food containers; equal or faster heating resulted when a water spray was used than when a waterbath or 100% steam was used.

In general, water-spray pasteurizers are more expensive in initial cost than steam-air pasteurizers because of the need for pumps and water reservoir tanks and are thought to have higher maintenance costs. The heating rates in water-spray pasteurizers are equal to, or higher than, those in a steam-air pasteurizer, and a properly operated water-spray pasteurizer eliminates some of the temperature distribution problems that occur in steam-air tunnels.

Continuous Agitating Atmospheric Cookers

The continuous agitating atmospheric cooker is widely used for the processing of acid foods such as fruits, fruit juices, and tomato products. The can conveying section is identical to that of the continuous agitating pressure cooker (spiral cooker), in that the cans travel along a spiral track as they proceed through the unit. The difference is that the shell is not pressurized and, therefore, the heating medium is maintained at atmospheric pressure. These systems are used with both steam at atmospheric pressure, normally near 212°F (100°C), and as water cookers. A rather simple mechanism, instead of a pressurized valve, is used to feed cans into the unit. The atmospheric cooker is considerably less expensive than the continuous agitating pressure cooker because of the simpler feed mechanisms and the use of an open-topped tank.

3.5.4 Heat Treatment of Bulk Foods before Aseptic Packaging

It is much faster to heat and hold (until pasteurization or sterilization is accomplished) and cool a food product that can be pumped through a system, then to pack it aseptically, than to heat treat a product after it has been sealed in a container. The three major heating systems used are heat exchangers, steam injection, and ohmic heaters.

Heat Exchangers

Under a given set of conditions, the rate of heating of a product in a heat exchanger depends on the distance the heat has to flow (rate of heat flow varies inversely with the square of the thickness) and the degree of agitating or mixing. In general, *heat exchanger* is the name given to a device designed to allow heat to flow from one fluid to another as economically as possible. The distance the heat must flow is made small, and the rate of fluid flow of both the heating medium and the product are as high as economical pumping will permit because short heat flow distances and high velocities produce the highest heat transfer rates. The chief limiting factor is that the product must be of such a consistency that it can be pumped through the heat exchanger.

Common types of heat exchangers are (1) *tubular heat exchangers* in which round or flattened tubes are used and the product normally flows through the tubes, with the heating medium surrounding them; (2) *plate heat exchangers* in which a series of plates is built up with appropriate openings and gaskets so that the heating medium is on one side of each plate and the product is on the other. Plate heat exchangers have a great amount of surface in relation to their physical size and are now available in designs that permit heating to 250°F (121°C), using steam as the heating medium, and are of such construction that the food products can be used as either the heating or the cooling medium; (3) *scraped-surface heat exchangers* are large diameter, tubular heat exchangers in which there is a mechanical scraping device to help agitate the product and keep product next to the wall continuously scraped and mixed with the remainder of the product. Scraped surface heat exchangers have very large heat transfer coefficients.

Steam Injection

Direct injection heating units, such as the Vacreater or the Fitzpatrick Calefactor, have large heating capacities for their physical size. Assuming that the product is at 150°F (65.5°C), and the designed temperature is 250°F (121°C), then 1 pound of steam will heat approximately 10 pounds of product. However, 1 pound of water will be added to the product. The advantage of this system is obvious in that the heat energy is injected directly into the product, and there is merely an equilibrium to be reached, which is the new temperature. The weight of steam that is added, of course, condenses to water. However, this is normally removed in a flash tank where the heated product, after having been held long enough to achieve sterilization, is flashed to a low pressure that will bring it back to the original temperature through the evaporation of a quantity of water approximately equal to that added to heat the product. When using direct steam injection heating, the processor must take adequate precautions to guard against the addition of impurities contained in the steam. There are heat exchange units now available where purified, softened, hot tap water can be converted into steam from the central boiler as a heat source. In this way, the processor has direct control over the material added to the product.

Ohmic (Electrical Resistance) Heating

Ohmic is the name given to an electrical resistance heating food product machine. The basic principle is that an alternating current voltage is imposed, through a pair of electrodes, on an electrically conducting food product with the result that heat is produced in proportion to the voltage imposed and the resistance of the food product. Resistance heating is an old technology. What is new is the engineering development of a system that makes it practical to use the system to heat food. Systems have been developed for continuously heating pumpable products prior to aseptic packaging (Figure 3-10). The developers of the present systems indicate that it can be used to heat particles up to about 25 mm in diameter. An advantage of the process is that for particles of uniform electrical resistance, there will be uniform heating throughout the particle. Because the input to the processing system is electrical energy, the energy cost may be higher than for systems where fossil fuels are used as the energy sources. The advantage of the ohmic over the steam injection system is that particles will be heated uniformly throughout rather than requiring heat to flow from the outside of the particle to its center by conduction heat transfer, as is the case if the particles are being pumped through a heat exchanger.

The rate of heating is a function of the power put into the system. Heating is dependent on the electrical conductivities of the raw materials. This type of heating should be considered when it is necessary to increase the rate of heat penetration into particles in pumpable liquids, and also when it may be desirable to decrease the length of the heating period. There is no similar system for increasing the rate of heat flow out of a product after it has been heated, so cooling has to be carried out in conventional heat exchang-

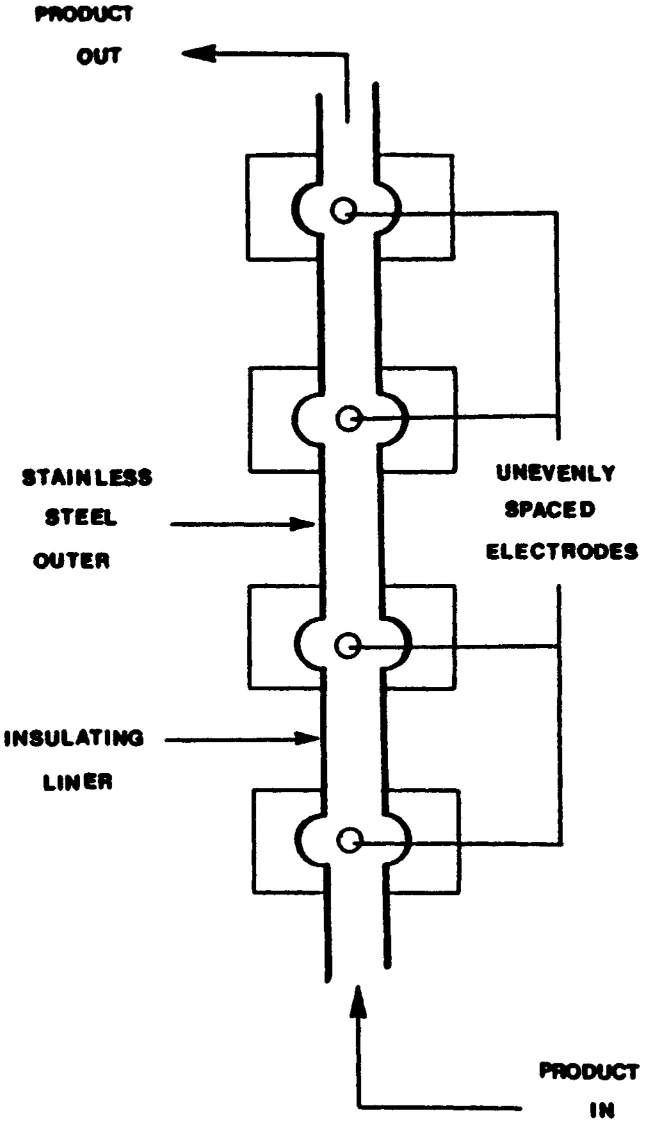


Figure 3-10 APV Ohmic Heater. *Source:* Reprinted from P.S. Richardson and J.D. Selman, Heat Preserving Equipment, in *Processing and Packaging of Heat Preserved Foods,* J.A.G. Rees and J. Bettison, eds., © 1991, Aspen Publishers, Inc.

ers. The role of ohmic heating in aseptic processing was summarized by Dignan et al.[38] and Fryer.[46]

Microwave Heating

Microwave cooking is heating by radio frequency excitation of water molecules in a food. There are no special killing effects from the radio frequencies used.[67] Kill is determined by the food temperature just as it is in other forms of cooking, though the overall effectiveness may be impaired by uneven heating. Microwave energy is used to heat some types of products on a continuous basis, where the product has been filled into a flexible package and is then heated in a microwave tunnel, sealed, and distributed as a chilled food with an extended shelf life.

In general, ohmic heating is most usefully applied to liquid systems whereas microwave heating is more applicable to solid foods. Both microwave and ohmic heating systems at the plant site use, as energy input, electricity, whereas heat exchanger systems, using steam or water or steam injection, can use fossil fuel energy sources, which may make the energy less expensive.

3.5.5 Aseptic Packaging

Aseptic packaging must be used if low-acid foods are sterilized in bulk. Puddings, juices, milk and milk products, vegetable soups, and purees are examples of the many products that are aseptically packaged.[90]

The Dole aseptic system, dating from about 1950, was probably the first major commercial aseptic system and is still in use. It was designed to use metal containers. From 1950 to 1980, there was continual progress in the development of aseptic packaging systems for bulk products, but little development for retail products. In the 1980s, there was a resurgence of interest in aseptic processing because of two major developments: (1) in the US, the Food and Drug Administration (FDA) approved the use of hydrogen peroxide for sterilization of aseptic packaging materials[4] and (2) form-fill-seal technology, using paperboard laminates to aseptically pack juices and other high-acid products was introduced.[89]

A large number of package types are now used in aseptic systems. These include metal cans, composite cans, thermoformed cups, tubs and trays, plastic laminated pouches, paper-plastic foil cartons, blow-molded bottles of many different shapes and sizes, plastic bags inside cardboard boxes, and very large stainless steel or epoxy-lined rigid tanks. In general, a specific type of package sterilization and aseptic filling and sealing equipment exists for each type of container system.

For ***form-fill-seal processes***, the packaging material arrives at the processing plant as roll stock. This can be mechanically formed into the container, which is then filled and sealed, or it can be thermoformed, ie, a cup or tray shape is drawn in the roll stock, the cavity filled, and a cover is sealed in place, all in an aseptic environment.

Fill-seal systems are those in which preformed containers arrive at the plant. Usually they are sterilized as part of the aseptic system then, after sterilization, they are aseptically filled and sealed.

In ***erect-fill-seal systems***, the packaging material arrives as a knocked down container. In the aseptic machine, it is formed or erected, sterilized, and then aseptically filled and sealed.

Another method is to ***blow-mold*** the container, then fill and seal. The extrusion temperature of the plastic is sufficiently high that a blow-molded container is sterile at the time it is produced. Consequently, the only requirement is to maintain sterility during filling with sterile product and sealing.

The surface of the package and closure and the aseptic transfer zone (the gas atmosphere and the surfaces of that part of the system where product, package, and closure are brought together), must all be sterile. Many methods are used.

In the Dole system, superheated steam is used to sterilize the containers and covers, and to sterilize the filler and closing machine during operation. Superheated steam is not as efficient as saturated steam as a bactericidal agent because it is dry heat. However, if the temperature is high enough, it will sterilize a clean, dry surface.[40,43,72,73] The advantage of superheated steam over saturated steam is that it is at atmospheric pressure, thereby eliminating the necessity of valving the containers into and out of a pressurized sterilization chamber.

Where the containers are made of plastic and/or paper, most aseptic systems use hydrogen peroxide. However, steam may be used to sterilize the metal surfaces that enclose the aseptic transfer zone. Packages and packaging materials also may be sterilized by ionizing radiation or other chemicals. Peracetic acid, ethylene oxide, halogens, ozone, and orthophosphoric acid have all been considered and/or used, singly and in combinations. Surface sterilization was reviewed by Cousin.[36]

Aseptic equipment must be designed so that (1) the designated sterile zone can be sterilized prior to processing, (2) packaging material can be sterilized, (3) the operation produces a shelf-stable, packaged product, and (4) all presterilizing and sterilizing functions are closely controlled. When the packaging material is sterilized, in whole or in part, by immersion in a liquid bactericide, an automatic sensing device must be included to ensure an adequate level of the bactericide.

Air and/or gasses used in the designated sterile zone and in contact with food product, packaging material, and/or sterile surfaces must be (1) approved (FDA approved in the US) for the intended use, and (2) filtered and incinerated, or filtered through at least two separate systems, each capable of producing sterile air.

Improved quality of aseptically processed food products is often achieved by taking advantage of the difference in thermal destruction rates between bacteria and food quality factors. Another advantage is that aseptic containers will usually be less expensive than conventional containers, will often be rectangular in dimension so that they stack more efficiently, and will usually weigh less than a conventional can or glass jar.

The present state of aseptic technology is (1) that there are few problems with acid foods, (2) systems are in place that will handle homogeneous low-acid products,[92] and (3) systems are being constructed, in 1998, for low-acid products containing particulates. However, aseptic is a complex industrial technology where there are still several technological and validation hurdles that need to be addressed before sustained growth of this industry can be assured.[16,30,38,44,77,104,110,114]

3.5.6 Hot Filling

Hot-filled food products with pH <4.6 are heated in some type of continuous system to a temperature and time that are adequate to effect microbial control for the specific product. The hot product is filled into containers and the containers sealed. The containers may only be washed before filling, or they may be washed and decontaminated before filling. After filling and sealing, the containers of hot product may be held before cooling, to effect microbial control of container and closure.[100] Using this system, a product such as tomato juice is heated in a closed system to a temperature of 240°–250°F (115°–121°C), and then cooled to about 210°F (99°C). The product is filled into containers at a temperature at or above 190°F (88°C), the container is then sealed, held hot for 1 to 3 minutes and cooled. The same general procedure is recommended for most fruit juices such as apricot, grapefruit, orange, and pineapple, which are normally heated to about 190°F (88°C) in a plate-type heat exchanger, filled into cans or bottles, making sure that the closing temperature is at least 175°F (79°C), sealed, held for a few minutes, and then rapidly cooled. If glass containers are used, a holding conveyor is required, which is usually part of the cooling system. To minimize thermal shock breakage, the cooler must have a tempering zone where the water temperature is about 130°F (54.5°C) in addition to the final cooling zone where the water temperature is 60°–80°F (15.5°–26.7°C).

An air blast is used to dry jars if they are to be labeled immediately. The product should be cooled to 100°F (38°C) before warehousing. An excellent summary is given by the National Canners Association.[69]

A system named "flash 18" has been developed for filling and sealing containers in a pressurized room held at about 18 psi gauge, making it possible to apply higher temperature, hot fill processing to low-acid foods. In this system, the food may be heated as high as 255°F (124°C) in a heat exchanger, filled, and sealed in metal or glass containers at this temperature in the pressurized room, then rapidly cooled. Heat damage during in-container cooling is only slight compared with heat damage to sensitive foods during in-container heating in large (No. 10) containers. The system has obvious advantages for packing sterilized nonacid foods in large, hermetically sealed containers.

3.6 CONCLUDING REMARKS

In this chapter the kinetics of heat destruction of microorganisms, methods of measuring moist-heat resistance, selecting F_T-values for low-acid canned foods, and the delivery of heat in commercial microbial control processes have been described.

In the the treatment of foods for microbial control, we have a science operation and a technology operation that must be combined in order to deliver safe and stable food. The science was covered in the first part of the chapter and the technology was emphasized in the latter part. It is perhaps important to mention that the scientist seeks to determine the truth regarding an area under study, and usually in a small well-defined area, whereas the technologist is trying to produce safe food in a system where he does not necessarily know the values of all the variables. In the selection and delivery of adequate processes, technologists must know their products and use judgment in selecting the critical parameters. We have tried to include some guidelines in this area. An expanded treatment can be found in Pflug et al.[75,77,78,80]

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Appendix 3-A

Definitions

F_T:	***Equivalent heating time at heating medium temperature.*** The equivalent time at the medium heating temperature, T, provided by the heat process calculated using a specified z-value.
F_0:	***Sterilization equivalent process time.*** Number of equivalent minutes at T = 250°F delivered to a container of product calculated using a z-value of 18°F (or at T = 121.1°C with a z-value of 10°C).
L:	***Lethal rate.*** The rate of microbial destruction at temperature T expressed in terms of the reference temperature, T_{ref}. The units of the lethal rates are minutes at T_{ref} per minute at T. $L = 10^{[(T-Tref)/z]}$.
N_0:	***Initial number of microorganisms per unit.*** The initial number of microorganisms per unit, sometimes called the bioburden.
N_U, N_F:	***Number of microorganisms per unit after stress.*** The number of viable microorganisms per unit that survive a heat process of U minutes at T_1 or of F minutes at temperature T.
TDT, F_T:	***Thermal death time.*** The time at temperature T necessary to reduce the number of microorganisms in a homogeneous population by a specified amount. $F_T = Y_n \times D_T$.
U:	***Equivalent minutes at heating-medium temperature.*** Net or equivalent time at the heating-medium temperature, T_1. $U = F(T_1,z)$.
z-value:	***Temperature coefficient of microbial destruction.*** The negative reciprocal of the slope of the TDT, TR, or RKT curves. The number of degrees of temperature change necessary to cause the F-, D-, or RKT-value to change by a factor of 10, measured in degrees F or C.

4 Irradiation

Margaret F. Patterson and Paisan Loaharanu

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4.1 INTRODUCTION AND HISTORICAL PERSPECTIVE

Radiation processing is not a new technology and has been used for many years for the sterilization of medical supplies and for the treatment of plastics to improve their properties. The application of the technology for the treatment of food is also not new. A United Kingdom (UK) patent was issued in 1905 for the use of ionizing radiation "to bring about an improvement in the condition of foodstuffs," and in 1921 the US Department of Agriculture (USDA) Bureau of Animal Industry proposed the use of x-rays for inactivating trichinae in pork.[24] However, it was not until the 1950s that sufficient quantities of suitable isotopes were available for the treatment of food. This development, together with the initiation of an "Atoms for Peace" program by the late President D. Eisenhower in 1953, led to an intensified research program to evaluate the potential applications of food irradiation in the 1950s and 1960s in both advanced and developing countries. However, the safety for consumption of irradiated foods became an issue for concern in the late 1950s due to the US Food Additive Amendment of the federal Food, Drug and Cosmetic Act, which classified food irradiation as a "food additive." Such a classification resulted in an international effort to prove the wholesomeness of irradiated food, initially carried out in advanced countries such as the United States (US), the UK, Germany, The Netherlands, and Japan.

In 1970, an International Project in the Field of Food Irradiation (IFIP) was established in Karlsruhe, Germany, under the aegis of the Food and Agricultural Organization of the United Nations (FAO), the International Atomic Energy Agency (IAEA), and the Organization for Economic Cooperation and Development (OECD) with participation by 24 countries, to investigate the wholesomeness of irradiated food. The World Health Organization (WHO) became associated with the International Project in an advisory capacity. This research effort, coordinated by IFIP, generated a significant amount of data on wholesomeness of irradiated food for evaluation by a Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food (JECFI), which was convened in 1976 and again in 1980. In October 1980, when the data on long-term animal feeding studies of irradiated food, and on the radiation chemistry of foods and food constituents were available, the JECFI was able to reach a conclusion that "the irradiation of any food commodity up to an overall average dose of 10 kiloGray (kGy) presents no toxicological hazard; hence, toxicological testing of foods so treated is no longer required." The expert committee also found that irradiation up to 10 kGy "introduces no special nutritional or microbiological problems."[1] The conclusions of the JECFI report, along with information available from national expert committees, led the Joint FAO/WHO Codex Alimentarius Commission to adopt the Codex General Standard for Irradiated Foods and the Recommended International Code of Practice for the Operation of Radiation Facilities for the Treatment of Food in 1983.[2] This view was reaffirmed in 1994 by WHO with the statement that "irradiated food produced in accordance with established good manufacturing practices can be considered safe and nutritionally adequate."[8]

These positive conclusions have provided major milestones in the further development of food irradiation. As a result, 38 countries including the US, France, the UK, South Africa, China, The Netherlands, and Thailand have approved the irradiation of many different food items or classes of food[6] (Appendix 4-A). However, because of policy and economic reasons, not all of these countries are using the technology in practice. Currently, 35 countries are using the technology for treating different types of foods (Table 4-1).

The wholesomeness of food irradiated with doses above 10 kGy was evaluated by a Joint FAO/IAEA/

Table 4-1 List of Irradiation Facilities Available for Food Processing

Country	*Location (starting date for food irradiation)*	*Products*
Algeria	Mascara	Potatoes
Argentina	Buenos Aires (1986)	Spices, spinach, cocoa powder
Bangladesh	Chittagong (1993)	Potatoes, onions, dried fish
Belgium	Fleurus (1981)	Spices, dehydrated vegetables, deep frozen foods
Brazil	Sào Paulo (1985)	Spices, dehydrated vegetables
	Piracicaba Manaus	Fruits, vegetables, grain
Canada	Laval (1989)	Spices
Chile	Santiago (1983)	Spices, dehydrated vegetables, onions, potatoes, poultry meat
China	Chengdu (1978)	Spices and vegetable seasonings, Chinese sausage, garlic
	Shanghai (1986)	Apple, potatoes, onions, garlic, dehydrated vegetables
	Zhengzhou (1986)	Garlic, seasonings, sauces
	Nanjing (1987)	Tomatoes
	Jinan (1987)	Not specified
	Lanzhou (1988)	Not specified
	Beijing (1988)	Not specified
	Tienjin (1988)	Not specified
	Daqing (1988)	Not specified
	Jianou (1991)	Not specified
	Beijing (1995)	Rice, garlic, spices
Croatia	Zagreb (1985)	Spices, food ingredients, dried beef noodles
Czech Republic	Prague (1993)	Spices, dry food ingredients
Cuba	Havana (1987)	Potatoes, onions, beans
Denmark	Riso* (1986)	Spices
Finland	Ilomantsi (1986)	Spices
France	Lyon (1982)	Spices
	Paris (1986)	Spices, vegetable seasonings
	Nice (1986)	Spices/herbs
	Vannes* (1987)	Poultry (frozen deboned chicken)
	Marseille (1989)	Spices, vegetable seasonings, dried fruit, frozen frog legs, shrimp, poultry (frozen deboned chicken)
Germany	Munich (1997)	Spices
Hungary	Budapest (1982)	Spices, onions, wine cork, enzyme

continues

Table 4-1 Continued

Country	*Location (starting date for food irradiation)*	*Products*
India	Bombay	Spices
	Nasik	Onions, potatoes
	Vashi, New Bombay	Spices
Indonesia	Pasar Jumat (1988)	Spices, rice
	Cibitung (1992)	
Iran	Tehran (1991)	Spices
	Yazd* (1997)	Dried fruits, nuts
Israel	Yavne (1986)	Spices, condiments, dry ingredients
Italy	Bergamo* (1996)	Spices
	Padoa*	Spices
Japan	Shihoro (1973)	Potatoes
Korea, Republic	Seoul (1986)	Garlic powder, spices, condiments
Malaysia	Bangi (1997)	Spices, herbal and dried food ingredients
	Penang (1999)	Spices
	Kedah (1999)	Spices
Mexico	Mexico City (1988)	Spices and dry food ingredients
Netherlands	Ede (1981)	Spices, frozen products, poultry, dehydrated vegetables, egg powder, packaging material
Norway	Kjeller (1982)	Spices
Peru	Lima (1996)	Spices, food additives, animal feed
Poland	Wlochy* (1991)	
South Africa	Durban (1989)	Spices
	Pretoria (1968)	Shelf-stable food
	Kempton Park (1982)	Spices
	Cape Town (1986)	Fruits, spices
Thailand	Patumthani (1989)	Onions, fermented pork sausages, enzymes, spices
UK	Swindon (1991)	Spices
US	Rockaway, NJ (1984)	Spices
	Whippany, NJ (1984)	Spices
	Tustin, CA (1984)	Spices
	Ames, IA* (1993)	Spices, poultry
	Mulberry, FL (1992)	Fruits, vegetables, poultry, spices
	Schaumberg, IL	Spices
	Columbus, OH	Spices
	Morton Grove, IL	Spices, fruits
	Haw River, NC	Spices
	Salem, NJ	Spices
	Hilo, HI (1998)	Tropical fruits
Vietnam	Ho Chi Minh City (1999)	Spices, dried herbs
Yugoslavia	Belgrade (1986)	Spices

Note: Facilities underlined are under construction or planned; countries underlined are irradiating food for commercial use. (All facilities use Co-60 as radiation source except those indicated by *, which are electron beam facilities.)

Courtesy of International Atomic Energy Agency, Vienna, Austria.

WHO Study Group in September 1997. Having examined data on toxicological, nutritional, microbiological, and radiation chemistry aspects of foods so treated, the Group concluded that food irradiated to any dose appropriate to achieve the intended technological objective is both safe to consume and nutritionally adequate. There is no need for an upper dose limit to be imposed. Thus, irradiated foods are deemed wholesome throughout the technologically useful dose range below 10 kGy to envisioned doses above 10 kGy.[137]

4.2 IRRADIATION PROCESSING

A commercial irradiation facility consists of an irradiation chamber that contains the source of the ionizing radiation, a conveyor system that passes the products into and out of the radiation chamber, and separate loading and unloading areas and a control unit (Figure 4-1). The major structural difference between this type of plant and other industrial buildings is the thick concrete walls (typically 2 m) that surround the irradiation room. These walls act as a shield and prevent the radiation from penetrating beyond the chamber.

Only certain radiation sources can be used for the treatment of foods. These are the radionuclides cobalt-60 and caesium-137, which emit gamma rays; electron beam machines, which emit electrons with a maximum energy of 10 million electron volts (MeV); and x-ray machines, which have a maximum energy of 5 MeV. The radiation emitted from all the sources is referred to as "ionizing" because the energy is high enough to dislodge electrons from atoms and molecules and to convert them to electrically charged particles called ions. However, the energies from these sources are too low to induce radioactivity in any material, including food. Of the radionuclides, only Co-60 is available in sufficient quantities for commercial irradiation. In the case of the radionuclides, radiation is constantly emitted and, when not in use, the source is stored in a deep (typically 6 m) water pool. Machine sources, however, are electrically operated and can be switched on and off.

The conveyor system, which transports products around the radionuclide source, follows a convoluted pathway to ensure a more even dose distribution. The separate loading and off-loading areas ensure that untreated and treated products are kept separate. The speed of the conveyor system (and hence, the dose delivered) is preselected at the control unit. The unit also allows the monitoring of the radiation source and the processing conditions from outside of the irradiation chamber.

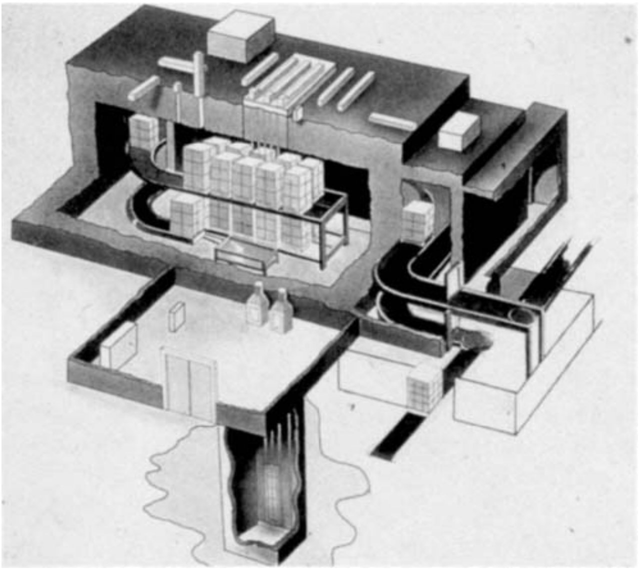

Figure 4-1 Automatic Pallet Irradiation Facility. Courtesy of Gammaster B.V., Ede, The Netherlands.

Worldwide there are approximately 160 commercial gamma irradiation facilities and hundreds of electron accelerator facilities used mainly to process nonfood products. These facilities are strictly monitored to ensure their safe operation and are subject to government legislation and control. Irradiation facilities typically cost 1 million to 3 million US dollars or more depending on size and capacity.[65] Therefore, medical supplies are often sent to a central commercial facility. It is likely that food companies would also use central facilities for the irradiation of their products.

4.3 APPLICATIONS OF FOOD IRRADIATION

Treatment of food with ionizing radiation can produce a wide variety of beneficial effects, including a delay in ripening of fruits and vegetables; inhibition of post-harvest sprouting of tuber and bulb crops; and the destruction of insects, parasites, pathogenic and nonpathogenic bacteria, yeasts, and molds. This chapter is concerned primarily with the microbiological effects of food irradiation. Further information on the other applications can been found elsewhere.[58,132]

The amount of radiation absorbed by a food, the absorbed dose, is measured in grays (Gy), where one gray is equal to the absorption of 1 Joule/kg. In most food applications, the dose required is defined in kilograys (kGy). Irradiation treatments aimed at the inactivation of microorganisms can be divided into three classes:

1. ***Radappertization:*** the treatment of a food with a sufficient minimum dose of irradiation, usually 25 to 45 kGy, to produce a commercially sterile product. This is equivalent to the sterility achieved with heat processing (canning). Foods treated in this way can be stored for several years at ambient temperatures, provided they are not recontaminated. High dose food applications include the provision of sterile diets for immunocompromised individuals. Irradiation sterilized foods have also been used by US astronauts, by defense force personnel, and by specific recreation and sport groups such as hikers, campers, and yachters in South Africa.

2. ***Radicidation:*** the treatment of a food with a dose of irradiation sufficient to reduce the numbers of viable, non–spore-forming pathogenic bacteria to undetectable levels using standard bacteriological procedures. This is equivalent to pasteurization with heat and requires doses of <10 kGy. This treatment will also inactivate foodborne parasites such as tapeworm and trichina in meat.
3. ***Radurization*:** the treatment of a food with a dose of radiation (<10 kGy) sufficient to reduce numbers of spoilage microorganisms and so enhance the keeping quality of foods.

Radicidation and radurization treatments will not kill all microorganisms, therefore, the food will eventually spoil unless preserved in some other way.

4.4 BIOLOGICAL EFFECTS

If the death rate is exponential, the sensitivity of a microorganism to irradiation can be defined as a D_{10}-value, the dose (kGy) required to inactivate 90% of the population. If N_0 is the initial number of the organisms present, and N is the number remaining after irradiation with a dose D then

$$D_{10} = \frac{D}{\log N_0 - \log N}$$

In many cases, a linear relationship is obtained in a semi-logarithmic plot of log survivors against dose, so the D_{10} can be calculated as –1/slope of the regression line. In other cases, there is a "shoulder" at lower doses or a residual "tail" at higher doses. Bacteria exhibiting a shoulder in their inactivation curves possess more than one vital target per cell, while all targets should be hit to inactivate the cell. The shoulder can also represent the ability of an organism to repair some DNA damage so that more than one hit is required to inactivate the cell, while tailing is most often observed in complex media such as fish meal and crabmeat.[86] When the inactivation curves are not linear, it is difficult to accurately estimate a D_{10}-value. Alternatively, the minimum required dose (MRD) to achieve *n* log cycles reduction can therefore be calculated. In practice, the MRD can be the dose of irradiation that, at a given initial contamination level, reduces the final numbers to a nondetectable level, ie, the minimum required dose. This approach has been used to calculate the dose required to achieve a 10^{12} reduction of *Clostridium botulinum* spores in certain foods under specified conditions (see below).

The radiation sensitivities of representative microorganisms are given in Tables 4-2 through 4-5. In general, Gram-negative bacteria are more sensitive than Gram-positive ones and rods are more sensitive than cocci. Yeasts and molds tend to be more resistant than bacteria and bacterial spores are even more resistant. Viruses are generally the most resistant of the microorganisms. Many interacting factors can affect the sensitivity of microorganisms to irradiation and, as illustrated in Table 4-2, this can lead to very different D_{10}-values, depending on the treatment conditions, as illustrated below.

Intracellular factors include the nature and extent of the damage to a vital target, usually DNA, and the ability of the cell to repair this damage. When ionizing radiation is absorbed by any living biological system, there is the possibility that it will act directly on the DNA and initiate a chain of events, which may ultimately lead to cell death. This effect can cause single and double stranded breaks in the DNA within the cell. Most microorganisms have the ability to repair at least some DNA damage and this in part is reflected in their radiation resistance.[84] Some bacteria, such as *Deinococcus radiodurans* and *Deinobacter grandis* have developed particularly extensive repair mechanisms and are extremely radiation resistant. However, these bacteria do not appear to have any public health significance and are not important in causing spoilage. Alternatively, the radiation may interact with other molecules in the cell, particularly water, to produce highly reactive entities such as the hydrated electron, ·OH, ·H, H_2O_2, and H_3O^+, which can damage the DNA. These intermediates are very short lived and interact with the chemical components of the food and produce stable end-products. The end-products are similar to those found naturally in foods or to compounds produced by heat processing. This indirect effect is of most importance in vegetative cells because they have a relatively high water content.[24]

Viruses have relatively little nuclear material and are orders of magnitude smaller than bacteria and so, being smaller "targets," are relatively resistant to irradiation (have higher D_{10}-values). This also explains why bacteria are more resistant to radiation than protozoa, parasites, and insects. The DNA in mammalian cells contain much more genetic information than these organisms and is correspondingly larger and more sensitive to radiation. The structural arrangement of the DNA itself can also affect radiation sensitivity. Bacterial cells, which are actively dividing (in the exponential phase of growth), are usually more sensitive than those in the lag or stationary phases.[56]

Table 4-2 Radiation D_{10}-Values (kGy) for Bacteria

Organism	D_{10}-*Value (kGy)*	*Substrate*	*Temperature (°C)*	*Atmosphere and Other Remarks*	*Reference*
Acinetobacter calcoaceticus	4.05–8.14	beef	–30		78
Aeromonas hydrophila	0.21	shrimp paste	–20	vacuum	105
	0.11–0.15	bluefish	22		97
	0.22–0.34	bluefish	–15		
	0.11–0.15	beef	2		
Bacillus cereus (vegetative cells)	0.17	roast beef	3–4	air	43
	0.18	gravy			
	0.21	cauliflower			
	0.20	mashed potato			
	0.18	mechanically	5	logarithmic phase cells	126
	0.42	deboned chicken		stationary phase cells	
Bacillus cereus (spores)	2.46	meat (MDCM)		spores (all samples in air)	
Campylobacter coli	0.12–0.25	chicken	3–4	air	101
Campylobacter fetus	0.14	chicken	3–4	air	101
Campylobacter jejuni	0.14–0.16	ground beef	18–20	microaerophilic	122
	0.32	ground beef	–30	air	66
	0.16	ground beef	0–5		
	0.17	ground beef	30		
	0.24	low (8%) fat beef	–16	air	22
	0.18	low (8%) fat beef	4		
	0.13–0.19	chicken	3–4	air	101
Campylobacter lari	0.14	chicken	3–4	air	101
Clostridium botulinum (spores)	3.45–3.60	cooked beef	25	type A spores	46
	3.73–3.85	cooked beef	0		
	4.30–4.34	cooked beef	–50		
	5.77–5.95	cooked beef	–196		
	1.37	beef stew	room temp.	type E spores	11
Clostridium perfringens (vegetative cells)	0.59	roast beef	3–4	air; D_{10} calculated from exponential region of curve	43
	0.41	gravy			
	0.53	cauliflower			
	0.35	mashed potato			
	0.83	pork	10	air	41
	0.75	pork		25% CO_2; 75% N_2	
Clostridium sporogenes	6.3	beef fat	—	air	113
	7.8	pork fat			
	10.10	chicken fat			
	4.7	soybean oil			
Deinobacter species	5.05	pork	15	air	39
Deinobacter grandis	3.6	phosphate buffer		—	96
Deinococcus radiodurans	2.5	raw beef	5	—	29
	2.4	chicken			
	3.39	fish			

continues

Table 4-2 Continued

Organism	D_{10}-*Value (kGy)*	*Substrate*	*Temperature (°C)*	*Atmosphere and Other Remarks*	*Reference*
Escherichia coli 0157:H7	0.26	MDCM	0	air; cells from stationary phase	125
	0.27	MDCM	0	vacuum, cells from stationary phase	
	0.16	MDCM	0	vacuum, cells from log phase	
	0.44	MDCM	–5	vacuum, cells from stationary phase	
	0.27	beef	0	vacuum, cells from stationary phase	
	0.31	low (14%) fat beef	–16	air	22
	0.24	low (14%) fat beef	4		
	0.31	high (27%) fat beef	–16		
	0.25	high (27%) fat beef	4		
Escherichia coli (nonpathogenic)	0.35	chicken	4	air	99
	0.29	chicken		CO_2	
	0.27	chicken		vacuum	
	0.23	chicken		N_2	
	0.39	surface of prawn	–10	air	49
Lactobacillus species	0.59	chicken	4	air	99
	0.40	chicken		CO_2	
	0.50	chicken		vacuum	
	0.51	chicken		N_2	
Lactobacillus sake	1.47	beef	—	air	48
	1.08	beef		CO_2	
	1.34	beef		vacuum	
	1.95	beef		N_2	
Listeria monocytogenes	0.49	chicken	12	air	99
	0.57–0.65	pork	10	air	41
	0.62–0.71	pork		25% CO_2; 75% N_2	
	0.27–0.46	MDCM	2–4	air	54
	2.0	ice cream	–78	—	47
	0.59	low (14%) fat beef	4	air	83
	0.61	low (14%) fat beef	–16		
	0.57	high (27%) fat beef	4		
	0.58	high (27%) fat beef	–16		
	0.64	roast beef	3–4	air	43
	0.56	gravy			
	0.56	cauliflower			
	0.53	mashed potato			
Moraxella nonliquifaciens	5.39–5.83	beef	–30		78
Moraxella osloensis	4.77–10.00	beef	–30		78
Moraxella phenylpyruvica	0.86	chicken	4	air	99
	0.68	chicken		CO_2	
	0.63	chicken		vacuum	
	0.88	chicken		N_2	
Pseudomonas fluorescens	0.13	low (%) beef	5		79
Pseudomonas putida	0.08	chicken	4	air	99
	0.11	chicken		CO_2	
	0.06	chicken		vacuum	
	0.08	chicken		N_2	

continues

Table 4-2 Continued

Organism	D_{10}-*Value (kGy)*	*Substrate*	*Temperature (°C)*	*Atmosphere and Other Remarks*	*Reference*
Salmonella enteritidis	0.70	low (3.3%–5.6%) fat beef	—	air	79
	0.49	high (40%–44%) fat beef			
	0.48	surface of prawn	–10	air	49
	0.30–0.45	shrimp		frozen	90
Salmonella typhimurium	0.64	low (3.3%–5.6%) fat beef	5	air	77
	0.53	high (40%–44%) fat beef			
	1.74	fish meal	—	air	71
	0.63	liquid egg			
	0.49	egg albumen			
	0.57	roast beef	3–4	air	43
	0.42	gravy			
	0.59	cauliflower			
	0.46	mashed potato			
		pork	10	air	41
				25% CO_2; 75% N_2	
	0.50	chicken	4	air	99
	0.54	chicken		CO_2	
	0.62	chicken		N_2	
	0.62	low (13%) fat beef	2–5		22
	0.80	low (13%) fat beef	–17		
	0.66	high (28%) fat beef	2–5		
	0.75	high (28%) fat beef	–17		
	0.55	ground beef	18–20	air	122
	0.37	filet americain			
	0.60	soft-shell clam		—	72
	0.63	mussels			
Shigella flexneri	0.35	soft-shell clams	—	—	72
	0.34	mussels			
Staphylococcus aureus	0.42	chicken	4	air	99
	0.41	chicken		CO_2	
	0.40	chicken		vacuum	
	0.37	chicken		N_2	
	0.45	low (11%) fat beef	4	air	83
	0.45	low (11%) fat beef	–16		
	0.45	high (28%) fat beef	4		
	0.44	high (28%) fat beef	16		
	0.39	roast beef	3–4	air	43
	0.36	gravy			
	0.43	cauliflower			
	0.42	mashed potato			
	0.88	soft-shell clam			72
Streptococcus faecalis	0.65	chicken	4	air	99
	0.70	chicken		CO_2	
	0.70	chicken		vacuum	
	0.68	chicken		N_2	
	1.0	clam	—		72
	1.05	mussels			
Vibrio cholerae	0.11	surface of prawn	–10±2	air	4
Vibrio mimicus	0.75	shrimp paste	–20	vacuum	105

continues

Table 4-2 Continued

Organism	D_{10}-Value (kGy)	Substrate	Temperature (°C)	Atmosphere and Other Remarks	Reference
Vibrio parahaemolyticus	0.04–0.06	shrimp homogenate	frozen		12
Vibrio vulnificus	0.30	shrimp paste	–20	vacuum	105
Yersinia enterocolitica	0.20	beef	25	air	31
	0.39	beef	–30		
	0.10–0.20	beef	18–20	air	122
	0.16–0.20	pork	10	air	41
	0.18–0.19	pork		25% CO_2; 75% N_2	

The radiation sensitivity of microorganisms also depends on extracellular conditions such as the nature of the substrate, temperature, and atmosphere during irradiation. Proteins can exert a protective effect on microorganisms. This is because of the presence of free radical scavengers, such as sulphydryl groups, which "mop up" water and oxygen radicals. For example, it has been reported that *Salmonella enteritidis* has a D_{10} of 0.70 kGy and 0.49 kGy in low-fat and high-fat beef, respectively.[79] Assuming that low fat means high protein, then it is possible that the radical scavenging properties of the protein in the low-fat, high-protein beef gave higher protection to the pathogens. For this reason, it is good practice to determine the radiation sensitivities of microorganisms in the actual foods of interest rather than extrapolate data from one type of food to another. If the food has a high water content, then the indirect action of radiation is important, and the bacteria have a lower D_{10}-value than in dry foods. Therefore, higher doses are generally needed to produce the desired microbial kill in dry foods such as spices. A similar argument applies to frozen foods. The water phase is immobilized so the radicals are unable to move and cause indirect damage. Again, higher doses are needed to produce the same microbial kill as in nonfrozen products.[124] The D_{10} of *Escherichia coli* O157:H7 in chicken meat was found to be 0.27 kGy at +5°C and 0.42 kGy at –5°C.[125] Such effects must be taken into account when deciding on an effective radiation dose.

The presence of oxygen generally increases radiation sensitivity. The oxygen molecule has unpaired electrons, and it can add on to many radicals, thereby preventing radical recombination and dimerization. Thus, the presence of oxygen can enhance the indirect action of radiation and result in a lower D_{10}-value. *S. senftenberg* in phosphate buffered saline at room temperature was reported to have a D_{10}-value of 0.13 kGy in aerobic conditions and 0.39 kGy under anoxic conditions. Similarly, *S. typhimurium* had an aerobic D_{10}-value of 0.21 kGy and 0.62 kGy under anoxic conditions.[129] However, the presence of O_2 during irradiation can also cause undesirable changes in sensory properties of the food, such as the oxidation of lipids. This has led to studies of the inactivation achieved in modified atmospheres. For example, some lactobacilli are reported to have lower D_{10}-values when irradiated in 100% CO_2 compared to air, vacuum, or N_2.[48] Studies on the outgrowth of *C. botulinum* type A and B spores and subsequent botulinum toxin production have also shown that low dose irradiation in the absence of O_2 act synergistically to delay toxin production in pork even at a moderate abuse storage temperatures of 15°C.[67]

The microorganisms that survive the irradiation process are likely to be damaged. In one study *Listeria monocytogenes* was inoculated into raw, sterile poultry meat at high initial levels so that detectable numbers would survive the irradiation treatment. The recovery and growth of the surviving cells was monitored during storage at different temperatures. The lag phase duration at 6°C was 1 day in unirradiated samples, 10 days in samples irradiated to 1 kGy, and 15 days in samples irradiated to 2.5 kGy. It was concluded that even if low numbers of *L. monocytogenes* were to survive irradiation treatment, the time required to recover from the irradiation damage would be significant, and growth of this pathogen would not be a problem during the shelf life of the poultry meat.[102]

The ultimate survival of damaged cells depends on postirradiation conditions. Thus, in order to get a valid estimate of survivors, the composition of the plating medium is important. Nutritionally simple media are better than rich media for the recovery of damaged cells.[84] Selective media may not allow all damaged cells to grow, so cells can appear to be more radiation sensitive than when recovered on nonselective media. For example, the D_{10} for *L. monocytogenes* in poultry meat was reported as 0.64 kGy when cells were enumerated on tryptone soya yeast agar and 0.52 kGy on the Listeria selective agar (Oxford formulation).[100] Similarly a suboptimal incubation temperature is better than the optimal. These conditions reduce growth rate and allow the cells time to repair damage.

4.5 IMPROVEMENT IN FOOD SAFETY

It is estimated that the biological contamination of food and water supplies is causing 1 billion cases of disease as well as about 5 million deaths annually.[59] Many of these cases occur in developing countries and are related to infant diarrhea, but industrialized countries also have significant levels of foodborne disease. The Centers for Disease Control and Prevention have estimated that the number of cases of salmonellosis in the US is between 800,000 and 4 million annually.[5] It has been estimated that only 10% of cases of foodborne disease are ever reported so the true figure is likely to be much higher.[106] An upward trend in the number of cases has been found in many countries. The majority of cases are due to either *Salmonella* or *Campylobacter* species. Foodborne cases from other pathogens such as *L. monocytogenes* and *E. coli* O157:H7, although relatively unimportant numerically, are also causing concern due to the high morbidity and mortality resulting from the illnesses they cause.[26]

The first of the 10 World Health Organization (WHO) Golden Rules for Safe Food Preparation advises the consumer to purchase foods processed for safety.[59] Examples quoted include the recommendation to consumers to buy pasteurized as opposed to raw milk and, if they have a choice, to select fresh or frozen chickens that have been treated with irradiation. Only 1 of the 10 rules refers to irradiation, so it is obvious that this technology cannot be a panacea to all problems of food safety but the WHO stresses that it makes a significant contribution.

4.5.1 Pathogenic Non–Spore-Forming Bacteria

Vibrio species and *Aeromonas hydrophila* are among the most radiation sensitive of the vegetative pathogens (Table 4-2). *V. parahaemolyticus* was found to have a D_{10}-value of 0.06 in frozen shrimp homogenate.[12] A dose of 1.0 kGy would therefore give at least a 10^{15} reduction in numbers. Palumbo et al.[97] reported D_{10}-values of 0.14 to 0.19 kGy for five strains of psychrotrophic *A. hydrophila* in ground beef irradiated at 2°C. No significant differences in radiation sensitivity were observed between stationary and logarithmic phase cells.

Yersinia enterocolitica is also very sensitive to irradiation with D_{10}-values of 0.10 to 0.20 kGy when treated in unfrozen foods. As expected, it is more resistant at lower temperatures. For example, in beef at 25°C, the D_{10} was 0.20 kGy compared to 0.39 kGy at –30°C.[31] *Campylobacter* species were found to have similar D_{10}-values to *Y. enterocolitica*, although there were significant differences in the radiation sensitivities between different *Campylobacter* species and within strains of the same species when treated in poultry meat at refrigeration temperatures.[101] However, even the most resistant strain, a pork liver isolate of *C. coli*, with a D_{10}-value of 0.25 kGy, was more sensitive than *S. typhimurium* and *L. monocytogenes* irradiated in poultry meat under similar conditions.

Undercooked hamburger meat and dairy products made from unpasteurized milk have been linked to outbreaks of hemorrhagic diarrhea due to the presence of *E. coli* O157:H7 (see Chapter 43). The organism has also been isolated from raw beef, poultry, pork, and lamb.[27] *E. coli* O157:H7 is relatively sensitive to radiation with D_{10}-values of approximately 0.27 kGy in chilled beef and chicken and 0.3 to 0.45 kGy in meats irradiated at –5° to –15°C.[22,125] Irradiation would, therefore, be an effective method to control this pathogen.

Foods implicated in intoxications due to *Staphylococcus aureus* are frequently cooked, or otherwise treated, for example with salt, so that the competitive microflora has been removed or inactivated. They become contaminated with the pathogen as a result of manual handling and, if subjected to temperature abuse, enterotoxin can be formed during storage. For example, the components of cook-chill ready meals are usually prepared individually, manually portioned and then stored at 3°C for up to 5 days (including day of production and consumption). If the meal components are contaminated with *S. aureus* during portioning, and temperature control is not strictly observed, the potential for toxin formation exists. It has been proposed that irradiation of the prepared meals, after portioning, would be an effective way of ensuring products free from *S. aureus* and other pathogens.[80] A dose of 2 kGy was found to be sufficient to ensure the safety of a roast beef ready meal without significantly affecting the sensory quality. However, irradiation should not be used as a substitute for Good Manufacturing Practice and only foods of good microbial quality should be irradiated. No pathogen should be present initially in meals at levels greater than about 100 g^{-1}.[4] Staphylococcal enterotoxin itself is extremely resistant to irradiation and heat. In a 15% ground beef slurry, 16% to 26% residual staphylococcal enterotoxin activity could be detected after a dose of 23.7 kGy.[108]

Salmonella species and *L. monocytogenes* are the most radiation resistant of the common vegetative pathogens with typical D_{10}-values of 0.50–1 kGy, depending on the substrate and radiation conditions (Table 4-2). Thus, doses recommended to give a significant reduction in numbers of these pathogens from foods will also be appropriate for other vegetative pathogens. For example, a dose of 3.0 kGy applied to fresh, chilled poultry meat would be expected to give at least a 10^4 reduction in numbers of *Salmonella* spp, and *L. monocytogenes*, a 10^9 reduction in *E. coli* O157:H7, and a 10^{12} reduction in *Campylobacter* species and *Y. enterocolitica*. It has been reported that Camembert cheese made from unpasteurized milk can also be successfully irradiated to 2.5 kGy, sufficient to give a significant reduction in numbers of *L. monocytogenes* and *Salmonella* species, provided that the storage temperature and ripening conditions are carefully controlled.[17] This is contrary to the idea that fatty foods, such as some fish and dairy products, are unsuitable for treatment due to the radiation-induced oxidation of the fats.

4.5.2 Pathogenic Spore-Forming Bacteria

The endospores of spore-forming bacteria are more resistant to most preservation treatments, including irradiation, than vegetative cells. Spores of *C. botulinum* are of most concern in shelf-stable foods. In thermal sterilization, the 12D concept is used to ensure foods are processed for safety. If this is applied to radappertized foods, then the dose must be high enough to reduce viable spores of the most resistant strain of *C. botulinum* by a factor of 10^{12}. Type A and B spores are regarded as being the most resistant of the *C. botulinum* spores to radiation while type E are most sensitive.[132] Thus, the MRD would be 12 times the D_{10}-value of the most resistant type A spore. Using the examples given in Table 4-2 for beef, this would be equivalent to a dose of 43.2 kGy at 25°C, 46.2 kGy at 0°C, and 52.08 kGy at –50°C. However, the theoretically determined MRD may not be what is required in practice.

In many cases there is not a simple straight-line relationship between dose and the logarithm of the surviving fraction of spores. For example, there may be a "shoulder" at the low dose end of the curve and a resistant "tail" at the high dose end.[86] D_{10}-values calculated from the linear portion of the curve will then not reflect the true resistance of the spores. Radappertized foods are frequently given a mild heat treatment sufficient to inactivate enzymes, and additional preservatives such as salt or nitrite may be present, depending on the product. All of these will influence the survival of the spores or their ability to germinate and produce toxin, and have to be taken into account when determining the radiation MRD. For these reasons, inoculated pack studies containing spores in the food of interest and prepared under specific conditions can be used instead of theoretical D_{10} calculations. Using this technique, 12D values have been calculated for various foods. For example, the MRD for beef was found to be 41.2 kGy, 26.9 kGy for corned beef, 43.7 kGy for pork, and 31.4 kGy for ham.[57] These values were calculated using a specific preparation technique, which included an enzyme-inactivating heat treatment to an internal temperature of 70°–80°C, vacuum packaging, and an irradiation temperature of –30°C. The addition of salt and nitrate and/or nitrite can influence the survival of the spores as can been seen in the different MRDs required for the cured and uncured meats.

Although the spores are generally present in low numbers, the potential problem of *C. botulinum* may also exist in foods treated with low doses of radiation. When assessing the botulinum risk in these foods, factors such as the probability of spores being present in the product prior to treatment, the dose to be applied, and the likelihood of removing sufficient spoilage microorganisms before toxin is elaborated, particularly if the product suffers temperature abuse, have to be considered. The possibility that the removal of the more radiation-sensitive microflora may, because of the reduced competition, encourage the growth of and toxin production by *C. botulinum* has been investigated. Type E and some of the nonproteolytic type B spores are of particular concern as they can grow and produce toxin in foods at temperatures as low as 3.3°C (Chapter 41). Type E *C. botulinum* is most frequently associated with fish and fishery products although the incidence is variable depending on geographical location.[132] The issue of microbiological safety which arises is whether recognizable sensory spoilage occurs prior to toxin formation in irradiated products. This question is not unique to irradiation but is also relevant to other nonsterilizing preservation treatments, such as the vacuum packaging of smoked fish or heat pasteurized crab meat. When making recommendations for the irradiation of fishery products it is clear that a number of factors must be considered. Some species of fish seem to support the growth of and toxin production by *C. botulinum* type E better than others. For example fatty fish contain ribose and glucose, which are essential for the low temperature growth of *C. botulinum* type E.[132] Exclusion of O_2, through vacuum packaging, may be desirable to reduce rancidity development in fatty fish. However, it also prevents the growth of aerobic spoilage bacteria and encourages the growth of anaerobes, including *C. botulinum*. The initial level of spores can have a significant effect on the time to toxin development. Many laboratory experiments used inoculation levels many orders of magnitude higher than would be encountered naturally. It has been shown that the time required for toxin production increases significantly as the spore population is decreased.[132] In many instances, temperature control below 3.3°C is an important safety feature.

In a series of experiments, fish fillets of different species were inoculated with *C. botulinum* type E and stored under various combinations of packaging, irradiation dose, and storage temperature. The samples were analyzed periodically for toxin production and spoilage off-odors.[30] It was found that the storage temperature had a significant effect on the results. In the case of haddock fillets, at 5.6°C toxin production always lagged behind spoilage by a substantial margin, irrespective of inoculum level (up to 10^4 spores g^{-1}) and irradiation dose (up to 2 kGy). At 7.8°C, however, the safety margin between spoilage and toxin formation was reduced at 2 kGy with 10^2 spores g^{-1}, and with a higher inoculum of 10^4 spores g^{-1}, toxin production preceded spoilage. In this case, packaging the fillets in O_2-permeable or impermeable film did not influence the results. When the experiment was repeated using petrale sole fillets (initial inoculum level = 10^2 spores g^{-1}), in impermeable film, it was found that at 3.3°C there was a large margin of safety between the appearance of spoilage odors and toxin production. However, at 5.6°C and 2 kGy, toxin was present before obvious spoilage. It would therefore appear that petrale sole is a better substrate than haddock for toxin production by *C. botulinum* type E.

In North America, the incidence of *C. botulinum* in red meat is generally low with an estimated 0.04 to 2.18 spores kg^{-1}.[51] The incidence in Europe appears to be higher and nonproteolytic type B is more common.[25] The incidence of *C. botulinum* in poultry meat is also considered to be extremely low and poultry products are very rarely associated with human botulism. However, the possibility that type E spores may be present, as a result of fish meal being included in the poultry diet, has also been considered. It has been shown that when poultry skin, moistened with poultry exudate, was inoculated with type E spores and irradiated to 3 kGy, the *C. botulinum* survivors could not compete with the natural surviving microflora. The samples were stored at 10°C and 30°C and spoilage off-odors were observed before toxin was produced.[35] In another study,[127] vacuum canned commercial mechanically deboned poultry meat was inoculated with 20 strains of *C. botulinum* type A and proteolytic B spores (400 g^{-1} meat). Samples were either not irradiated or irradiated at 5°C to 1.5 or 3.0 kGy and stored at 5°C for 0, 2, and 4 weeks. Toxin was not detected in any of the samples, although all became toxic within 18 hours at 28°C. The aerobic and/or facultative mesophilic counts reached the designated spoilage level of 10^7 cfu g^{-1} at 2 weeks in the control samples, between 2 and 4 weeks in the 1.5 kGy samples, and not at all after 4 weeks in the 3.0 kGy samples during storage at 15° and 35°C. Only the unirradiated samples were considered to have a spoilage odor after 4 weeks. Gram-positive bacteria, mainly streptococci, dominated the irradiated samples during storage and would likely give an acid-sour odor upon opening the vacuum packages.

It is well established that, like staphylococcal enterotoxin, extremely high doses of irradiation are required to inactivate botulinum toxin in food. The botulinum toxin activity remaining in ground beef mixtures irradiated to 25 kGy increased from 1% to 30% as the proportion of ground beef increased from 15% to 100%.[131] Rose et al.[108] also found that increased concentrations of beef slurry conferred increased protection to botulinum and staphylococcal toxins against irradiation.

Clostridium perfringens may be a contaminant of meat and poultry. The spores are resistant to irradiation and the vegetative cells appear to be more resistant than *Salmonella* species, when irradiated in meat substrates (Table 4-2). Radicidation doses proposed for the elimination of other vegetative pathogens may not give sufficient inactivation of this pathogen. Storage below 15°C is therefore necessary for controlling the growth of *C. perfringens* in irradiated foods, as is recommended for other nonsterilizing preservation treatments.

It has been reported that the radiation resistance of *B. cereus* spores is not affected by temperature, desiccation, or suspending medium to the same extent as vegetative cells.[75] A study of the effect of temperature during irradiation found that stationary phase vegetative *B. cereus* cells inoculated into sterile beef and irradiated to 3 kGy decreased in numbers by 4.20, 5.27, and 5.72 $\log_{10}$ units at -20°, 0°, and 20°C. However, irradiation temperature was found to have no significant effect on spore survival.[126] A comparison of the radiation response of six strains of *B. cereus* inoculated into pre-irradiation sterilized ground beef, pork, chicken, turkey, and a heat sterilized beef gravy was then made. The D_{10}-values obtained in mechanically deboned chicken for one strain were 0.18 (log phase), 0.45 (stationary phase), and 2.56 kGy (spores). Spores for all strains were more resistant in beef, pork, and gravy than in poultry meats. In another study[45] irradiation of roast beef and gravy to 2 kGy at 4°C resulted in a 10^3 to 10^4 reduction in numbers of vegetative *B. cereus* cells. During subsequent storage at an abuse temperature of 15°C, the surviving cells grew slower, possibly as a result of irradiation injury, and toxin production was inhibited although toxin was found in the unirradiated controls. Toxin was found in both nonirradiated and irradiated samples stored at 22°C.

4.5.3 Viruses

Food virology is a relatively new science, and there is still much research needed to determine the role of viruses in foodborne disease (Chapter 52). However, outbreaks of foodborne illness have been associated with viruses such as Norwalk and hepatitis A.[11] The inactivation rates of viruses by irradiation are exponential functions of the radiation dose.[24] D_{10}-values tend to be high, typically 2–7 kGy at irradiation temperatures up to 20°C. The D_{10}-value increases further as the temperature during irradiation decreases (Table 4-3). Thus, irradiation doses below 10 kGy will not give an adequate reduction in the numbers. Foods given low doses of irradiation may still contain infectious viruses. This is not unique as other nonsterilizing preservation treatments, such as freezing, will also allow the survival of viruses. Viruses can be inactivated by heat[56] so the combination of heating with irradiation can be used successfully.

4.5.4 Parasites

Foodborne parasitic infections are widespread in both advanced and developing countries but are often not recognized by health and regulatory authorities. These infections are frequently associated with consuming raw or underprocessed food of animal origin, and the resulting economic losses can be enormous (Chapters 50 and 51). For example, the cost of foodborne toxoplasmosis, caused by *Toxoplasma gondii*, in the US is estimated to be up to 2.6 billion dollars a year.[107] Worldwide, the rates of congenital toxoplasmosis infection are estimated at 0.1% to 0.8% of live births or up to about 1 million infected babies born each year.[14] Another parasitic disease caused by the liver fluke *Opisthorchis viverrini*, through consumption of raw freshwater fish, results in an estimated economic loss of up to 99 million dollars per annum to the society in Thailand.[74]

Foodborne parasites, in general, are more radiation sensitive than pathogenic microorganisms. Thus, radiation doses required to ensure the microbiological safety of food of animal origin appear to be adequate for controlling foodborne parasites as well. As foodborne parasitic infections normally occur in rural areas, it may be difficult to demonstrate the cost-effectiveness of irradiation as a public health control measure. However, where irradiation is used to ensure microbiological safety of food, it could be demonstrated that such a measure will also be effective for controlling infections caused by foodborne parasites.

It should be noted that radiation doses required for controlling infection of foodborne parasites do not necessarily kill them outright. In general, a radiation dose below 1 kGy will prevent the most infective stage of parasites from infecting humans. For example, a dose of approximately 0.5 kGy will prevent the tapeworms *Taenia saginata* and *T. solium* from developing the segments required for infection. A

Table 4-3 D_{10}-Values (kGy) of Viruses

Organism	*D_{10}-Value (kGy)*	*Substrate*	*Temperature (°C)*	*Atmosphere and Other Remarks*	*Reference*
Hepatitis A	2.02	clams/oyster		air	76
Poliovirus	3.1	oysters		air	76
	3.0	fish fillets		air	50
Rotavirus	2.4	clams/oysters		air	76
Coxsackie virus	7.5	raw beef	–30	air	121
	6.8		–90		
	6.8	cooked beef	–30		
	8.1		–90		

minimum dose of 0.3 kGy will sterilize the most infective stage of the nematode *Trichinella spiralis* without killing it. Radiation doses required for controlling infections of foodborne parasites are summarized in Table 4-4.

4.6 EXTENSION OF SHELF LIFE

On a global scale, between a quarter and a half of the world's food supply is lost post-harvest as a result of spoilage, insect infestation, and bacterial and fungal attack.[3] Irradiation is one method of reducing such losses.

4.6.1 Nonpathogenic Bacteria

Irradiation, like any other nonsterilizing treatment, has a selective effect on the heterogeneous microflora of foods. Many of the common spoilage microorganisms such as *Pseudomonas* species, and other aerobic Gram-negative bacteria are sensitive to irradiation, with D_{10}-values below 0.2 kGy (Table 4-2). Therefore, irradiation doses aimed at improving food safety will also be effective at reducing numbers of spoilage organisms, resulting in an extension of shelf life, particularly of flesh foods. Organisms in the *Moraxella/Acinetobacter* group are unusual in that, although they are Gram-negative, many of them are relatively resistant to irradiation, with D_{10}-values similar to yeasts (Tables 4-2 and 4-5). In general, however, there is a shift to a predominately Gram-positive microflora after irradiation.

Lactic acid bacteria are relatively resistant to irradiation (Table 4-2). Although they are generally present as a minor component of unirradiated fresh meats, they tend to dominate the microflora of irradiated meats, especially if packaged under a vacuum or in a CO_2-enriched atmosphere.[52] *Lactobacillus sake* and *L. curvatus* have been found to be the dominant types in both irradiated beef and pork.[42,48] Unlike most other bacteria, *L. sake* is reported to be more radiation resistant in the log growth phase than in the stationary phase, and more resistant when irradiated in N_2 or under vacuum than under CO_2.[48] The development of a lactic microflora may be desirable as it can give additional consumer protection. The lactic acid bacteria produce acidic metabolites that lower the pH of the environment, thus

Table 4-4 Effective Radiation Doses for Parasites

Parasite	*Minimum Effective Dose (kGy)*	*Bioassay Model*	*Reference*
Protozoa			
Toxoplasma gondii	0.5	mice, cats	28
	0.7	mice,cats	136
	0.45–0.6	mice, cats	118
Trematodes			
Fasciola hepatica	0.03	mice	23
Clonorchis sinensis	0.2	guinea pigs	20
	0.15	guinea pigs, rats	116
Opisthorchis viverrini	0.1	hamsters	119
Paragonimus westernami	0.1	cats	117
Cestodes			
Taenia saginata	0.4	human volunteers	130
	0.3	human volunteers	134
Taenia solium	0.2–0.6	hamsters	133
Echinococcus granulosus	0.5	mice	123
Nematodes			
Trichinella spiralis	0.3	mice	38
	0.3	rats	37
	0.15	mice, rats	18
	0.11	rats	63
	0.1–0.6	mice	61
Angiostrongylus cantonensis	2.0	mice	95
	4.0	mice	95
Gnathostoma spinigirum	7.0	rats	112
Anisakis species	6.0	*in vitro* (agar plate)	110

Table 4-5 Inactivation Dose (kGy) for Yeasts and Molds

Microorganism	*Irradiation Dose (kGy)*	*Substrate*	*Temperature (°C)*	*Atmosphere and Other Remarks*	*Reference*
Candida zeylanoides	0.68	chicken skin	10	air, calculated as D_{10}-value	53
Cryptococcus albidus	2.5	chicken skin	10	air, calculated as D_{10}-value	53
Cryptococcus albidus	10	grape juice	ambient	value is dose needed to prevent growth of 0.6–2.5 × 10^6 cells ml^{-1}	34
Sporobolomyces roseus	0.75	phosphate buffered saline	4	air, calculated as D_{10}-value	81
Sporobolomyces pararoseus	5.0	nutrient broth	ambient	value is dose needed to prevent growth of 0.6–2.5 × 10^6 cells ml^{-1}	34
Saccharomyces cerevisiae	18	grape juice	ambient	value is dose needed to prevent growth of 1.5–3.0 × 10^6 cells ml^{-1}	33
Torulopsis stellata	10	grape juice	ambient	value is dose needed to prevent growth of 1.5–3.0 × 10^6 cells ml^{-1}	33
Penicillium expansum	0.32	grain	10	air, calculated as D_{10}-value	93
Penicillium viridicatum	1.4	0.1% peptone + 0.1% Tween 80	ambient	value is dose needed to prevent growth of 4 × 10^6 conidia ml^{-1}	88
Aspergillus flavus	1.7	0.1% peptone + 0.1% Tween 80	ambient	value is dose needed to prevent growth of 4 × 10^6 conidia ml^{-1}	88
Aspergillus ochraceus	0.44	grain	10	air, calculated as D_{10}-value	93

discouraging the growth of other bacteria, including spore-forming and vegetative pathogens.[56] Many strains have also been shown to produce bacteriocins and other antimicrobial substances, which can retard the growth of pathogens.

Niemand et al.[91] reported that a dose of 2.5 kGy resulted in a greater than 3 $\log_{10}$ reduction in the total aerobic and anaerobic plate counts in chilled beef. It was observed that the lactic acid bacteria dominated the microflora in both air and vacuum irradiated packs and that Enterobacteriaceae, *Brochothrix thermosphacta* and the pseudomonads were not detected. The shelf life of the beef stored at 4°C was reported to be "well over 9 days when evaluated by bacteriological criteria."

Similarly, in another study, the microflora of irradiated (1.75 kGy) pork packaged in a modified atmosphere (MA) of 25% CO_2:75% N_2 was almost exclusively composed of lactic acid bacteria, predominantly *Lactobacillus* species after storage at 4°C.[40] Later studies confirmed that many of these *Lactobacillus* species were capable of producing bacteriocins active against *L. monocytogenes.*[19] The shelf life of the irradiated MA packed pork, as assessed by a trained sensory panel, was found to be 12 days at 4°C, compared to 8 days in the MA without irradiation, and 2 days if packaged in air without irradiation.[40]

An irradiation dose of 2.5 to 5 kGy has been recommended for poultry carcasses.[60,70] This should not significantly affect the organoleptic quality of the product but can extend the shelf life at chill temperatures from 6 to 14 days and greatly reduce the number of pathogens.[87] A dose of 2.5 kGy was also reported to give a 4-week extension at 0°–3°C to the shelf life of chilled lamb pieces.[103]

4.6.2 Yeasts

Yeasts reproduce primarily by budding. Radiation can kill the cells outright but lower doses are sufficient to prevent them from reproducing. Nonlinear curves, with an initial shoulder, are often found when plotting $\log_{10}$ numbers of survivors against dose.[81] For these reasons, the radiation sensitivity of yeasts may be expressed as the minimum required dose or effective dose rather than as a D_{10}-value.[132] The effective dose is related to the size of the initial population, and this should be stated when quoting such values (Table 4-5).

In general, yeasts are more resistant to irradiation than bacteria. If it is assumed that an inactivation dose of 18 kGy for *Saccharomyces cerevisiae* reduces 3 × 10^6 cells to zero (Table 4-5), then this corresponds to a D_{10}-value of 2.8 kGy, similar to that of *C. botulinum* type E spores. An irradiation dose of 2.5 kGy reduced numbers of yeasts on chicken breasts[70] from 5 × 10^2 cfu g^{-1} to 3.2 × 10 cfu g^{-1} and 1.5 kGy reduced numbers of yeasts in British sausage by 1 $\log_{10}$, while a dose of 3 kGy resulted in almost a 2 $\log_{10}$ reduction.[81] *Sporobolomyces roseus* was found to be more radiation sensitive than *Debaryomyces* *hansenii,* which in turn was more sensitive than *Candida zeylanoides* and *Trichosporon cutaneum.*

Yeasts have the potential to contaminate all foods but are generally only significant in the spoilage of fruit and vegetables, cereal grains, legumes, and baked products.[132] They are usually not a problem in meats and poultry because initial numbers are low and their slow growth rates at refrigeration temperatures prevent them from competing effectively with psychrotrophic bacteria.[136] However, yeasts tend to be more resistant to irradiation than bacteria, and they can be important in the spoilage of irradiated meat and meat products, such as sausages, stored at chill temperatures.

4.6.3 Fungi

Fungi may produce both hyphae and spores, and it is difficult to determine cell numbers and therefore difficult to determine radiation D_{10}-values. As with yeasts, one alternative is to report minimum required dose levels or the dose required to suppress the growth of a known number of cells (Table 4-5). *Fusarium* species and *Alternaria* species are generally more resistant to irradiation than *Penicillium* species and *Aspergillus* species.[93] *Target theory* models have been developed to explain irradiation resistance. The *multiple hit* theory assumes that the target, usually DNA, must be hit a number of times before the organism is destroyed. Spores of *Fusarium* species and *Alternaria* species are multicellular and, therefore, from the multiple hit theory, if one cell escapes damage the spore can still have the ability to germinate. This is in contrast to the unicellarity of *Aspergillus* species and *Penicillium* species.

Fungal spoilage can be a problem in most fruits. Fresh strawberries normally have a short shelf life due to the growth of microorganisms such as *Botrytis, Rhizopus,* and *Mucor* species.[82] A dose of 2 kGy with storage at 5°C can control fungal growth and give a shelf life of 14 days compared to <7 days in the unirradiated controls. Similarly, fungal spoilage in other fruits such as citrus, pomme fruits, and stone fruits can be controlled by 1 or 2 kGy.[132] However, sensory changes can occur in some fruits, even at these doses. The problem can be solved by combining low dose irradiation with a hot water dip. For example, some varieties of mangoes cannot be irradiated successfully above 1 kGy, which is insufficient to inhibit fungal spoilage. An effective treatment is a combination of irradiation to 0.75 kGy with a 5-minute dip in water at 50° or 55°C. This combination along with transport and storage at 11°C gives a shelf life of around 30 days, of which 1 week can be at ambient temperature. If applied to mature green mangoes, the combination will not only control fungal decay but will also delay ripening and inactivate the mango weevil, if present.[133]

Low dose irradiation of grains, usually below 1 kGy, is also used as a method for insect disinfestation but can, in addition, reduce the fungal population. However, there has been some debate regarding the effects of irradiation on fungal metabolism, particularly on mycotoxin production. Some reports indicate that irradiation can enhance the production of aflatoxin[10,104] while others suggest that it has no effect[15,115] or decreases it.[21] Similarly, ochratoxin A production by *A. ochraceus* was found to have increased in some experiments and decreased in others.[98] There are several explanations for the variability of the results. It is known that inoculum size has a significant effect on mycotoxin production. It has been demonstrated that a 10^4 reduction in the number of spores, caused by simple dilution or by irradiation, resulted in a two-fold increase in aflatoxin production by *A. parasiticus*[114] and up to a 12-fold increase in aflatoxin production by *A. flavus.*[92] Thus, it appears that toxin production is suppressed when the number of inoculated spores per unit volume of substrate exceeds a certain optimum, perhaps due to a changed ratio with spoilage organisms. Many of the experiments in which irradiation appeared to enhance aflatoxin production used large spore inocula, so control samples, with no dilution effect due to irradiation, may have exhibited suppressed mycotoxin production due to the inocula size.

In a field trial in India, naturally contaminated wheat was stored at 90% relative humidity at 28°C, conditions not unusual in that continent. The same or lower levels of aflatoxin production were found in the irradiated (0.2 kGy) samples compared to unirradiated controls.[15] In another study, the effects of irradiation (2 kGy) on ochratoxin A production by *A. alutaceus* were monitored.[16] The authors reported that ochratoxin A production was diminished if the fungus was inoculated into the grain prior to irradiation (2 kGy). However, if the grain was irradiated first and then inoculated with *A. alutaceus*, then toxin production was enhanced suggesting that reduction or elimination of competitive microflora allowed greater production of the mycotoxin than would occur in the presence of effective competition. The effects of repeated cycles of sublethal irradiation on several strains of *A. flavus* has been studied.[36] It was found that such treatment more frequently led to a complete loss of, or decrease in, aflatoxin production than to an increase.

As with the other microbial toxins, the mycotoxins are themselves resistant to irradiation. Aflatoxins in rice were found to be destroyed exponentially by irradiation, with aflatoxin B_1 and G_1 being more radiation sensitive than B_2 and G_2.[64] However, a dose of greater than 500 kGy was required to destroy even the most radiation sensitive, aflatoxin G_1. Ochratoxin, at concentrations of 5, 10, and 20 $\mu g\ ml^{-1}$ was stable to an irradiation dose of 75 kGy.[98] The trichothecenes deoxynivalenol and 3-acetyl deoxynivalenol also required greater than 50 kGy for their destruction in grains.[94]

4.7 COMBINATION TREATMENTS

Although irradiation is a very potent and useful antimicrobial process in its own right, the dose that can be applied to a particular food, and hence the extent of the antimicrobial kill, can be limited by undesirable sensory changes. The threshold dose above which these changes can be detected varies with different foods. For example a slight "irradiation flavor" was detected at 1.75 kGy in pork, at 2.5 kGy in chicken, lobster, beef, and shrimp, and at 6.25 kGy in lamb.[120] One approach to achieving optimum quality in both microbial and sensory terms is to use irradiation in combination with other preservation techniques. The use of combination treatments can result in enhanced preservative action and/or reduction in severity of one or all the treatments.

The combination of irradiation with heat was first suggested in the 1950s when synergistic effects were observed with a variety of biological systems including bacteria. More recently it has been suggested that irradiation followed by heating could be used to control spoilage and increase the microbiological safety of certain foods.[32] The order of the application can affect the level of microbial inactivation achieved. In most instances, the preferred order is first irradiation and then heating. An irradiation dose of 5 kGy is sufficient to sensitize clostridial spores to subsequent heating.[62] This was found to be of value in heat-treated spiced meats. Spores, frequently natural contaminants of spices, were sensitized during irradiation of the spices. The spices were then incorporated into luncheon meat, which was canned and heat treated to give a commercially sterile product. The heat treatment required in the canning process was less (the F_0 was reduced from 4.5 to 3.0) as the spores were more heat sensitive. This reduction in heating was reported to give better product quality and a saving in energy.

Irradiation can also sensitize vegetative cells to subsequent heating. Thayer et al.[128] investigated the effects of heat and ionizing radiation on *S. typhimurium* in mechanically deboned chicken meat and reported that irradiation to a dose of 0.9 kGy caused heat sensitization in the pathogen. The radiation-induced heat sensitization was found to persist for up to 6 weeks at 5°C. In a similar study, the combined effect of irradiation followed by heat on the destruction of *L. monocytogenes* in roast beef and gravy was investigated.[44] An irradiation dose of 0.8 kGy was sufficient to cause heat sensitization. The radiation-induced heat sensitivity persisted for up to 2 weeks at 3°C. This synergy between irradiation and heat, coupled with the persistence of the radiation-induced heat sensitization could be advantageous in irradiated foods such as raw meats, which are cooked before consumption, and in ready meals, which are reheated prior to consumption. The simultaneous application of irradiation and heat can have a significant effect on microbial inactivation, particularly in foods with a high water content.[132] This can be one method of inactivating the resistant spores such as *C. botulinum*, where there is a dramatic drop in D_{10}-values at temperatures near 100°C, but the practical difficulties make this an unlikely commercial option at this time.

Hydrostatic pressure, another physical treatment, during or following irradiation can also increase the inactivation of spores.[138] The D_{10} of *B. pumilus* spores irradiated in potassium phosphate buffer (pH 6.8) under a pressure of 1085 atmospheres (approximately 100 MegaPascals) was 0.32 kGy compared to a D_{10} of 2.7 kGy when the spores were irradiated at atmospheric pressure.

The use of irradiation combined with modified atmosphere packaging (MAP) has been reported by several authors.[41,69] Some meat products are packaged under modified atmospheres, usually with increased levels of CO_2, to increase shelf life. Although MAP can reduce the growth rate of some undesirable microorganisms, including spoilage and pathogenic bacteria, it generally does not kill them. When the atmosphere is removed (the pack is opened), there is an initial lag phase before microorganisms, such as the pseudomonads, begin to multiply again and ultimately spoil the meat. This combination of irradiation and MAP has potential as it could be possible to reduce the numbers of spoilage and pathogenic microorganisms by irradiation and suppress the growth of surviving microorganisms during storage, using MAP, without significantly affecting organoleptic quality.

Packaging of pork chops in a gas atmosphere of 25% CO_2:75% N_2 followed by irradiation at 1.75 kGy was found to significantly reduce the growth of microorganisms. A shelf life of 12 days at 4°C was obtained, compared to 3 days for unirradiated chops packaged in air and 8 days for unirradiated chops packaged in 25% CO_2:75% N_2. In sensory tests, panelists preferred the color and odor of the MAP/irradiated pork.[140] A series of inoculation studies, using a variety of vegetative pathogens, indicated that the combination of 25% CO_2:75% N_2 followed by irradiation to 1.75 kGy, significantly improved the microbiological safety of the pork.[41] Pork is more frequently associated with human botulism than other meats, implying that it is more frequently contaminated with *C. botulinum*. The combined effects of low dose irradiation, modified atmosphere packaging, and storage temperature on the ability of *C. botulinum* type A and B (proteolytic) strains to produce toxin in fresh pork have been investigated.[67] Five strains each of type A and B heat-shocked spores were inoculated onto pork loins at a level of approximately 10^3 spores g^{-1}. After 2 days at 25°C, toxin was detected at all dose levels (0, 0.5, and 1 kGy) and at all initial oxygen levels (0%, 10%, and 20%, in N_2). At 15°C, all samples packaged in 10% and 20% O_2 were toxic after 14 days. At 0% O_2, with an oxygen absorbent, it took 21 and 43 days, respectively, for 0 and 1 kGy samples to be toxic. No toxin was found in any samples stored at 5°C, even after 44 days. It was concluded that the enhancement of toxin production in the presence of oxygen at 15°C was likely the result of respiratory CO_2 production stimulating spore germination.

In a related study[68] with two levels of O_2 and CO_2 (0% and 20% O_2, in N_2) and two radiation doses (0 and 1 kGy), it was observed that 1 kGy delayed toxin production only in samples packaged under 20% CO_2. It took 2 weeks longer to detect toxin in packs with 20% CO_2:80% N_2 than with 20% O_2:80% N_2 and toxin production was faster with 20% O_2:80% N_2 than with 100% N_2. The authors speculated that the slower rate of toxin production in the absence of O_2 was due to lactic acid bacteria outcompeting *C. botulinum* vegetative cells. The fact that toxin production is more rapid in 20% O_2 (similar to the composition of air) and that sensory problems due to oxidative rancidity are reduced in anoxia, suggests that pork could be irradiated successfully and safely in vacuum packages.[9]

The combination of chemical preservatives with irradiation has been investigated by several authors. The addition of sodium chloride to foods can reduce the radiation dose requirement. When *C. botulinum* type A and B spores were inoculated into turkey frankfurters, sodium chloride combined with irradiation (5 and 10 kGy) was more effective at inhibiting toxin production than the combination of irradiation with potassium or magnesium salts.[13] An inoculum level of 450–500 spores g^{-1} with a sodium chloride concentration of 2.5% and storage temperature of 27°C resulted in nonirradiated samples becoming toxic within 4 days. However, similar samples irradiated to 5 kGy were toxic after 40 days, while samples given a 10 kGy treatment were not toxic at 50 days, the maximum incubation time of the experiment. Other salts can be used effectively in combination with radiation. Rowley et al.[109] demonstrated that a combination of 40 μg nitrite g^{-1} and 15 kGy was sufficient to prevent toxin formation in bacon inoculated with 2 spores g^{-1} of *C. botulinum* (10 strains of type A and B) during storage at 27°C for 60 days. The incidence of *C. botulinum* in bacon is normally 10^{-3} to 6.4×10^{-5} cells g^{-1}. The authors considered therefore that a level of 2 spores g^{-1}, as used in their experiments, gave a sufficient safety margin.

4.8 CONSUMER CONCERNS

4.8.1 Mutations

It is well established that irradiation can increase the rate of mutation in living organisms, including bacteria. In theory such mutations could be more pathogenic than parent strains. However, a survey of the scientific literature suggests that this is not the case.[55] Conversely, it has been found that radiation treatment can result in loss of virulence and infectivity. In many cases, irradiated microorganisms exhibit reduced fitness for their environment and become more sensitive to heat, drying, and antimicrobial agents.[24] These mutants are at a disadvantage and do not compete well with nonirradiated organisms that would be encountered in practical situations. Mutants, therefore, usually disappear unnoticed. It should be noted that irradiation is not unique in its ability to increase mutation rates. Thermal processing and some food preservatives can all increase mutation rates in microorganisms, yet there is no evidence that they have caused problems due to increased virulence.[85]

Another concern is that radiation-resistant mutants might develop, particularly as a result of repeated irradiation treatments.[89] It is possible to induce radiation resistance by subjecting bacteria to many cycles of extremely low dose irradiation (<0.1 kGy). In such experiments, survivors from a single irradiation treatment are allowed to reach high numbers and then reirradiated. This process is repeated many times and eventually survivors become more radiation resistant than the original population.[77] It was subsequently shown that salmonellae with this acquired resistance were weakened in other physiological characteristics, even to the point that some were less infective than the parent strain.[73] In practice, food is only irradiated once with a dose suffi-

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cient to achieve a technological objective, eg, >1 kGy for microbial control, so such circumstances should not occur in practice. Again, radiation is not unique in inducing resistance under specialized conditions. Heat resistance can be induced in salmonellae by repeated cycles of exposure to heat, but this has not caused problems in pasteurization plants.[24]

The possibility that radiation might change the properties of bacteria, particularly pathogens, so that they escape correct identification has been considered.[55] It has been found that the most characteristics remain unchanged after mutation, and they can be identified by the usual methods.

The use of irradiation to produce microbial strains for a particular industrial purpose is well known. For example certain strains of *P. notatum* have been developed that produce increased yield of penicillin.[24] Similarly, selected microbial strains are used in vitamin and amino acid production. In all these cases, the mutants were screened so that only desirable strains were selected, and recovery conditions carefully were chosen so that they could survive. In conclusion, mutants are unpredictable. There is no guarantee that a new pathogenic strain will not arise from a parent microorganism, irradiated or not. However, expert opinion is that such an event is most unlikely.[24]

4.8.2 Methods for Detecting Irradiated Foods

Methods for detecting irradiated foods and quantifying the absorbed dose are desirable. This would allow governments to enforce labeling legislation both for foods produced within their country and for imported products. In turn, this should increase consumers' confidence in their choice to purchase irradiated or nonirradiated food. If irradiated products are chosen, the ability to quantify the dose would ensure that the technical purpose of the treatment has been achieved; for example, that the correct dose was applied to chicken to ensure effective reduction in *Salmonella* and other pathogens.

The ideal detection method should be simple, inexpensive, rapid, reliable, require a relatively small sample size, allow for the detection of a wide range of doses, and not give false positives. In addition, it should have general application to a range of foods, allow the dose to be quantified, and be able to identify irradiated constituents in combination foods.[8] At present, such a perfect universal method does not exist and may not be available for some time in the future. The analytical problem is that most, if not all, chemicals formed as a result of irradiation are not unique, and are naturally occurring or are produced by other treatments such as thermal processing.

The changes in lipids after irradiation has led to the development of gas-liquid chromatographic (GLC) methods for detecting irradiated foods. Although many of the volatile products formed by irradiation are similar to those produced by heating and autoxidation, their distribution patterns vary. This is a promising method for identifying irradiated foods containing lipids such as meat, fish, and eggs. The method does not require specific expertise above that necessary for the routine use of GLC equipment.

Irradiation can result in the formation of free radicals although these are also generated by processes such as heating. Radicals resulting from irradiation give a unique signal when subjected to electron spin resonance (ESR). This technique could be used to quantify the dose in poultry meat by analyzing bone samples, in shrimp by analyzing the shell, and in strawberries by analyzing the achenes. Although the technique is well established, it requires expensive equipment and skilled operators.

Luminescence is another promising detection method. Luminescence is the emission of light when trapped energy is liberated either by the addition of a chemical (chemiluminescence, CL) or by heating (thermoluminescence, TL), when trapped charge carriers are released. Both CL and TL have been used successfully in identifying certain irradiated spices and vegetables.

Other methods being evaluated include inhibition of seed germination as a way of detecting irradiated citrus, the use of electrical impedance to detect irradiated bulbs and tubers, measurement of DNA fragmentation by microelectrophoresis, and the comparison of the aerobic plate count with direct staining, using the direct epifluorescent filter (DEFT) technique for herbs and spices. Detailed information on various detection techniques can be found elsewhere.[7]

4.9 CONCLUDING REMARKS

Treating food by ionizing radiation is an effective means of inactivating spoilage and pathogenic microorganisms and parasites. The safety and effectiveness of food irradiation as a food processing/preservation method have been recognized by the Codex Alimentarius Commission of the FAO/WHO Food Standards program when it adopted the Codex General Standard for Irradiated Foods and its associated Code of Practice in 1983. It is increasingly recognized by national authorities as a method to control foodborne diseases of solid food, especially those of animal origin, in the same manner as thermal pasteurization is recognized for liquid food. Its property as "cold pasteurization" process offers unique advantages for inactivating spoilage and pathogenic microorganisms in frozen food and food intended to be consumed raw or minimally processed, both of plant and animal origin. Similar to other substerilization processes of food, food to be processed by irradiation must adhere strictly to the Code of Good Manufacturing Practices (GMPs) and irradiated food must be handled according to GMPs.

Many national authorities have increasingly demanded that food be processed based on the principles of hazard analysis critical control point (HACCP) to ensure its safety. Unlike commercially pasteurized liquid food, which has thermal pasteurization as its critical endpoint treatment to ensure its safety, other foods in the raw or frozen state have no critical endpoint treatment to ensure safety. The critical control point for the latter type of food is passed on to consumers and those who are involved in food preparation to cook food properly to ensure its safety. Experience of foodborne disease outbreaks in many countries have demonstrated that improper food preparation and cross-contamination during the process of food preparation was largely responsible for these outbreaks. While recognizing the importance of food preparation to ensure food safety, irradiation appears to be the only effective critical control point under the HACCP principle to ensure safety of solid food at the point of sale or for those intended to be consumed raw or undercooked.

With the increasing trade liberalization following the successful conclusion of the GATT Uruguay Round followed by the establishment of the World Trade Organization in 1995, the role of irradiation as a method to ensure hygienic quality of food, especially those of animal origin, is likely to be more prominent and in some cases, eg, spices and other food ingredients, is likely to be used routinely by the food industry. It is therefore most important that every effort be made by governments, the food industry, consumer organizations, and the media to inform the public of the safety and benefits of food irradiation as an effective method to inactivate microorganisms in food.

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Appendix 4-A

Clearance of Item by Country

Country	Item Name	Code	Type of Clearance	Approval Date	Dose Max (kGy)
Argentina	Asparagus	1	unconditional	8/2/90	2
	Fruits (dried)	2	unconditional	12/9/92	1
	Garlic	6	unconditional	4/3/87	0.15
	Mushrooms	1	unconditional	8/2/90	3
	Onions	6	unconditional	4/3/87	0.15
	Potato	6	unconditional	4/3/87	0.15
	Spices	3	unconditional	12/9/90	30
	Spices	3	unconditional	12/9/90	10
	Strawberry	5	unconditional	3/4/87	2.5
	Vegetables (dried)	2	unconditional	12/9/90	1
Bangladesh	Chicken	3	unconditional	12/29/83	7
	Condiments	2	unconditional	12/29/83	1
	Condiments	3	unconditional	12/29/83	10
	Fish	3	unconditional	12/29/83	2.2
	Fish (dried)	2	unconditional	12/29/83	1
	Fish products	3	unconditional	12/29/83	2.2
	Frog legs	3, 5	conditional	12/29/83	7
	Mango	1, 2	unconditional	12/29/83	1
	Onions	6	unconditional	12/29/83	0.15
	Papaya	1, 2	unconditional	12/29/83	1
	Potato	6	unconditional	12/29/83	0.15
	Pulses	2	unconditional	12/29/83	1
	Rice	2	unconditional	12/29/83	1
	Shrimp	3, 5	conditional	12/29/83	5
	Spices	2	unconditional	12/29/83	1
	Spices	3	unconditional	12/29/83	10
	Wheat	2	unconditional	12/29/83	1
	Wheat products	2	unconditional	12/29/83	1
Belgium	Garlic	6	conditional	10/16/80	0.15
	Gum arabic	3	conditional	9/23/83	10
	Herbs	3	conditional	9/29/83	10
	Onions	6	conditional	10/16/80	0.15
	Potato	6	conditional	7/16/80	0.15
	Shallot	6	conditional	10/16/80	0.15

continues

Source: Courtesy of the International Consultative Group on Food Irradiation (ICGFI), IAEA, Vienna.

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Shrimp	3	conditional	11/30/88	5
	Spices	3	conditional	9/29/83	10
	Strawberry	5	conditional	7/16/80	3
	Tea, herbal	3	conditional	11/30/88	10
	Vegetables (dried)	3	conditional	9/29/83	10
Brazil	Avocado	2	unconditional	9/25/89	1
	Banana	2	unconditional	9/25/89	1
	Beans	2	unconditional	3/8/85	1
	Fish	5	unconditional	3/8/85	2
	Fish (dried)	2	unconditional	3/8/85	2
	Fish products	5	unconditional	3/8/85	2
	Guava	2	unconditional	9/25/89	1
	Lemon	2	unconditional	9/25/89	1
	Maize	2	unconditional	3/8/85	0.5
	Mango	2	unconditional	9/25/89	1
	Melon	2	unconditional	9/25/89	1
	Onions	6	unconditional	3/8/85	0.15
	Orange	2	unconditional	9/25/89	1
	Papaya	2	unconditional	3/8/85	1
	Persimmon	2	unconditional	9/25/89	1
	Pineapple	2	unconditional	9/25/89	1
	Potato	6	unconditional	3/8/85	0.15
	Poultry	3	unconditional	3/8/85	7
	Rice	2	unconditional	3/8/85	1
	Spices	3	unconditional	3/8/85	10
	Strawberry	5	unconditional	3/8/85	3
	Tomato	2	unconditional	9/25/89	1
	Wheat	2	unconditional	3/8/85	1
	Wheat flour	2	unconditional	3/8/85	1
Canada	Herbs	3	unconditional	10/3/84	10
	Onions	6	unconditional	3/25/65	0.15
	Potato	6	unconditional	11/9/60	0.15
	Spices	3	unconditional	10/3/84	10
	Vegetable seasonings (dried)	3	unconditional	10/3/84	10
	Wheat	2	unconditional	2/25/69	0.75
	Wheat flour	2	unconditional	2/25/69	0.75
China	Apple	5	unconditional	9/30/88	0.4
	Apricot	2	unconditional	2/23/94	1
	Cereal grains	2	unconditional	11/30/84	0.45
	Chicken (spiced)	3	unconditional	2/23/94	8
	Cooked meat products	3	unconditional	2/23/94	6
	Fruits (dried)	2	unconditional	2/23/94	1
	Garlic	6	unconditional	11/30/84	0.1
	Litchi	2	unconditional	2/23/94	0.5
	Mandarin	5	unconditional	2/23/94	0.1
	Mushrooms	1	unconditional	11/30/84	1
	Onions	6	unconditional	11/30/84	0.15
	Peanut	2	unconditional	11/30/84	0.4
	Pork	7	unconditional	2/23/94	0.65

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Potato	6	unconditional	11/30/84	0.2
	Sausages (Chinese)	3	conditional	11/30/84	8
	Sweet potato wine	10	unconditional	2/23/94	4
	Tomato	5	unconditional	2/23/94	4
Costa Rica (Note: All clearances in Costa Rica are specified on the basis of average absorbed dose.)					
	Chicken	3	unconditional	7/7/94	7
	Cocoa beans	2	unconditional	7/7/94	1
	Cocoa beans	3	unconditional	7/7/94	5
	Condiments	2	unconditional	7/7/94	1
	Condiments	3	unconditional	7/7/94	10
	Fish	3	unconditional	7/7/94	2.2
	Fish (dried)	2	unconditional	7/7/94	1
	Fish products	2	unconditional	7/7/94	1
	Fish products	3	unconditional	7/7/94	2.2
	Legumes	2	unconditional	7/7/94	1
	Mango	2, 5	unconditional	7/7/94	1
	Onions	6	unconditional	7/7/94	0.15
	Papaya	2	unconditional	7/7/94	1
	Potato	6	unconditional	7/7/94	0.15
	Rice	2	unconditional	7/7/94	1
	Strawberry	5	unconditional	7/7/94	3
	Wheat	2	unconditional	7/7/94	1
	Wheat products	2	unconditional	7/7/94	1
Croatia	Cereal grains	2	unconditional	6/21/94	1
	Cereal muesli	2	unconditional	6/21/94	1
	Cereal muesli	3	unconditional	6/21/94	10
	Egg (frozen)	3	unconditional	6/21/94	3
	Egg powder	3	unconditional	6/21/94	3
	Egg products (frozen)	3	unconditional	6/21/94	3
	Enzyme preparations	3	unconditional	6/21/94	10
	Fish	3	unconditional	6/21/94	5
	Frog legs	3	unconditional	6/21/94	8
	Fruit	1, 3	unconditional	6/21/94	3
	Fruit (dried)	2	unconditional	6/21/94	1
	Fruit (dried)	3	unconditional	6/21/94	10
	Fruit juices (frozen)	5	unconditional	6/21/94	4
	Garlic	6	unconditional	6/21/94	0.5
	Gum arabic	3	unconditional	6/21/94	10
	Meat (fresh)	5	unconditional	6/21/94	3
	Meat (frozen)	3	unconditional	6/21/94	7
	Mushrooms (dried)	2	unconditional	6/21/94	1
	Mushrooms (dried)	3	unconditional	6/21/94	10
	Onions	6	unconditional	6/21/94	0.5
	Pork carcasses or parts	7	unconditional	6/21/94	1
	Potato	6	unconditional	6/21/94	0.5
	Poultry (fresh)	3	unconditional	6/21/94	3
	Poultry (frozen)	3	unconditional	6/21/94	7
	Roots	6	unconditional	6/21/94	0.5
	Sausages (dry, semi-dry)	3	unconditional	6/21/94	5

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Seafood	3	unconditional	6/21/94	5
	Spices	3	unconditional	6/21/94	30
	Sterile meals	9	unconditional	6/21/94	45
	Tea, herbal	3	unconditional	6/21/94	10
	Tubers	6	unconditional	6/21/94	0.5
	Vegetables	1, 3	unconditional	6/21/94	3
	Vegetables (dried)	2	unconditional	6/21/94	1
	Vegetables (dried)	3	unconditional	6/21/94	10
Cuba	Animal blood (dried)	2	conditional	1/1/90	2
	Avocado	1	conditional	8/1/92	0.25
	Bacon	3	conditional	3/1/91	4
	Casings (hog)	3	conditional	10/1/88	7
	Cocoa (dehydrated)	2	unconditional	1/1/89	2
	Cocoa beans	2	unconditional	1/1/88	0.5
	Fish (dried)	2	unconditional	5/1/93	1
	Garlic	6	unconditional	4/1/87	0.08
	Mango	1	conditional	7/1/92	0.75
	Meat	3	conditional	8/1/91	5
	Meat products	3	conditional	3/1/90	4
	Onions	6	unconditional	4/1/87	0.06
	Potato	6	unconditional	4/1/87	0.1
	Seafood	5	conditional	1/1/91	3
	Sesame seed	2	unconditional	10/1/93	2
	Spices	2	unconditional	8/1/90	5
Czech Republic	Foods (dehydrated)	3	conditional	8/24/92	10
	Spices	3	conditional	8/24/92	10
Denmark	Herbs	3	unconditional	12/23/85	15
	Spices	3	unconditional	12/23/85	15
Finland	Spices	3	unconditional	11/13/87	10
	Sterile meals	9	unconditional	11/13/87	100
France	Animal blood (coagulated)	3	unconditional	12/4/86	10
	Animal blood (liquid)	3	unconditional	3/6/87	10
	Animal blood plasma (dried)	3	unconditional	12/4/86	10
	Animal blood products (dried)	3	unconditional	12/4/86	10
	Apricot (dried)	3	unconditional	7/25/91	6
	Camembert cheese	3	unconditional	3/23/93	3.5
	Casein, Caseinates	3	unconditional	7/21/91	6
	Cereal flakes	3	unconditional	6/16/85	10
	Cereal germ	3	unconditional	5/17/85	10
	Chicken	3	unconditional	9/1/90	5
	Chicken meat (mechanically separated)	3	unconditional	3/23/85	5
	Dates	3	unconditional	7/25/91	6
	Egg products	3	unconditional	11/17/90	4
	Figs (dried)	3	unconditional	7/25/91	6
	Frog legs	3	unconditional	5/8/88	8
	Fruit (dried)	2	unconditional	1/13/88	1
	Garlic	6	unconditional	7/12/84	0.15
	Gum arabic	3	unconditional	6/16/85	9
	Herbs	3	unconditional	5/22/90	10

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Onions	6	unconditional	7/12/84	0.15
	Poultry	3	unconditional	9/1/90	5
	Raisins	3	unconditional	7/25/91	6
	Rice flour	3	unconditional	11/13/88	5
	Shallot	6	unconditional	7/12/84	0.15
	Shrimp	3	unconditional	10/10/90	5
	Spices	3	unconditional	2/10/83	11
	Strawberry	5	unconditional	1/6/89	3
	Vanilla	3	unconditional	1/28/86	11
	Vegetables (dried)	10	unconditional	6/16/85	10
	Vegetables (dried)	2	unconditional	1/13/88	1
Hungary	Cherries	5	conditional	4/15/82	2.5
	Cherries, sour (canned)	10	conditional	2/20/84	0.2
	Chicken (frozen)	3	conditional	10/3/83	4
	Currants, red	5	conditional	4/15/82	2.5
	Grapes	5	conditional	4/15/82	2.5
	Mixed dry ingredients	3	conditional	11/20/76	5
	Mushrooms	1	conditional	4/15/82	3
	Mushrooms (*Agaricus* spp.)	1	conditional	4/15/82	2.5
	Onions	6	unconditional	6/23/82	0.2
	Pear	1	conditional	1/24/83	1
	Potato	6	conditional	1/28/83	0.1
	Spices	3	unconditional	8/19/86	6
	Strawberry	5	conditional	4/15/82	2.5
India	Onions	6	unconditional	8/9/94	0.09
	Potato	6	unconditional	8/9/94	0.15
	Seafood (frozen)	3	conditional	3/2/91	6
	Shrimp	3	conditional	3/2/91	6
	Spices	3	unconditional	8/9/94	14
Indonesia	Beans	3	unconditional	2/10/95	5
	Bulbs	6	unconditional	12/29/87	0.15
	Cereal grains	2	unconditional	12/29/87	1
	Fish (dried)	5	unconditional	2/10/95	5
	Frog legs (frozen)	3	unconditional	2/10/95	7
	Garlic	6	unconditional	12/29/87	1
	Onions	6	unconditional	12/29/87	1
	Potato	6	unconditional	12/29/87	1
	Pulses	3	unconditional	2/10/95	5
	Roots and tubers	6	unconditional	12/29/87	0.15
	Shrimp (frozen)	3	unconditional	2/10/95	7
	Spices	3	unconditional	12/29/87	10
Iran	Spices	3	unconditional	7/9/90	10
Israel	Animal feed	3	unconditional	2/17/87	15
	Cereal grains	2	unconditional	2/17/87	1
	Cocoa beans	2	unconditional	2/17/87	1
	Coffee beans	2	unconditional	2/17/87	1
	Edible seeds	2	unconditional	2/17/87	1
	Fruit	2	unconditional	2/17/87	1
	Mushrooms	5	unconditional	2/17/87	3

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Nuts	2	unconditional	2/17/87	1
	Poultry	3	unconditional	2/17/87	7
	Pulses	2	unconditional	2/17/87	1
	Spices	3	unconditional	2/17/87	10
	Strawberry	5	unconditional	2/17/87	3
	Vegetable seasonings (dried)	2, 3	unconditional	2/17/87	10
	Vegetables	2	unconditional	2/17/87	1
	Vegetables (dried)	3	unconditional	2/17/87	10
Italy	Garlic	6	unconditional	8/30/73	0.15
	Onions	6	unconditional	8/30/73	0.15
	Potato	6	unconditional	8/30/73	0.15
Japan	Potato	6	unconditional	8/30/72	0.15
Korea, Republic of	Chestnuts	2	unconditional	10/16/87	0.25
	Enzyme preparations	3	unconditional	5/19/95	7
	Fish powder	3	unconditional	12/14/91	7
	Garlic	6	unconditional	12/14/91	0.15
	Meat (dried)	3	unconditional	12/14/91	7
	Mushrooms	2	unconditional	10/16/87	1
	Mushrooms (dried)	2	unconditional	10/16/87	1
	Onions	6	unconditional	10/16/87	0.15
	Potato	6	unconditional	9/28/87	0.15
	Red pepper paste powder	3	unconditional	12/14/91	7
	Shellfish powder	3	unconditional	12/14/91	7
	Soy sauce powder	3	unconditional	12/14/91	7
	Soybean paste powder	3	unconditional	12/14/91	7
	Spices	3	unconditional	9/13/88	10
	Starch	3	unconditional	12/14/91	5
	Sterile meals	9	unconditional	5/19/95	10
	Vegetable seasonings (dried)	3	unconditional	5/19/95	10
	Vegetables (dried)	3	unconditional	5/19/95	7
	Yeast powder	3	unconditional	5/19/95	7
Mexico	Beef (dehydrated)	3	unconditional	4/7/95	10
	Bulbs	2	unconditional	4/7/95	0.2
	Cereal grains	2	unconditional	4/7/95	1
	Cereal products	2	unconditional	4/7/95	1
	Chicken (dehydrated)	5	unconditional	4/7/95	10
	Chicken (fresh or frozen)	3	unconditional	4/7/95	7
	Chicken (fresh or frozen)	5	unconditional	4/7/95	3
	Chicken products (fresh or frozen)	3	unconditional	4/7/95	7
	Chicken products (fresh or frozen)	5	unconditional	4/7/95	3
	Cocoa (dehydrated)	3	unconditional	4/7/95	5
	Condiments (dried)	3	unconditional	4/7/95	10
	Corn	2	unconditional	4/7/95	1
	Corn products	2	unconditional	4/7/95	1
	Egg (dehydrated)	3	unconditional	4/7/95	5
	Fish (fresh or frozen)	3	unconditional	4/7/95	5
	Fish (fresh or frozen)	5	unconditional	4/7/95	3
	Fish (fresh or frozen)	7	unconditional	4/7/95	2
	Food colors (natural, dehydrated)	3	unconditional	4/7/95	10

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Frog legs (fresh or frozen)	3	unconditional	4/7/95	5
	Frog legs (fresh or frozen)	5	unconditional	4/7/95	3
	Frog legs (fresh or frozen)	7	unconditional	4/7/95	2
	Fruit	1, 4	unconditional	4/7/95	1
	Fruit	5	unconditional	4/7/95	2.5
	Fruit (dried)	2	unconditional	4/7/95	1
	Fruit (dried)	3	unconditional	4/7/95	10
	Garlic	6	unconditional	4/7/95	0.2
	Herbs	2	unconditional	4/7/95	1
	Herbs	3	unconditional	4/7/95	10
	Mango	1, 4	unconditional	4/7/95	1
	Mango	5	unconditional	4/7/95	2.5
	Milk (dehydrated)	3	unconditional	4/7/95	5
	Mushrooms	1, 4	unconditional	4/7/95	1
	Mushrooms	5	unconditional	4/7/95	2.5
	Onions	6	unconditional	4/7/95	0.2
	Papaya	1, 4	unconditional	4/7/95	1
	Papaya	5	unconditional	4/7/95	2.5
	Pork	7	unconditional	4/7/95	1
	Potato	6	unconditional	4/7/95	0.2
	Rice	2	unconditional	4/7/95	1
	Rice products	2	unconditional	4/7/95	1
	Roots and tubers	6	unconditional	4/7/95	0.2
	Soup stock (dehydrated)	3	unconditional	4/7/95	10
	Soybean	2	unconditional	4/7/95	1
	Soybean products	2	unconditional	4/7/95	1
	Tea, herbal	3	unconditional	4/7/95	10
	Vegetables	1, 4	unconditional	4/7/95	1
	Vegetables	5	unconditional	4/7/95	2.5
	Wheat	2	unconditional	4/7/95	1
	Wheat products	2	unconditional	4/7/95	1
Netherlands	Cereal flakes	2	unconditional	8/1/92	1.5
	Frog legs	3	unconditional	8/1/92	7.5
	Fruit (dried)	2	unconditional	8/1/92	1.5
	Gum arabic	3	unconditional	8/1/92	15
	Herbs	3	unconditional	8/1/92	15
	Legumes	2	unconditional	8/1/92	1.5
	Poultry	3	unconditional	8/1/92	10.5
	Shrimp	3	unconditional	8/1/92	4.5
	Spices	3	unconditional	8/1/92	15
	Sterile meals (frozen)	9	unconditional	8/1/92	112.5
	Vegetables (dried)	2	unconditional	8/1/92	15
Norway	Herbs	3	unconditional	7/16/82	10
	Spices	3	unconditional	7/16/82	10
	Vegetable seasonings (dried)	3	unconditional	7/16/82	10
Pakistan	Garlic	6	unconditional	6/13/88	0.15
	Onions	6	unconditional	6/13/88	0.15
	Potato	6	unconditional	6/13/88	0.15
	Spices	3	unconditional	6/13/88	10

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
Philippines (Note: The clearance for spices specifies a minimum dose of 6 kGy but no maximum.)					
	Garlic	6	conditional	10/26/81	0.1
	Onions	6	conditional	10/26/81	0.1
	Spices	3	conditional	4/28/92	—
Poland	Garlic	6	unconditional	10/1/90	0.15
	Mushrooms (dried)	3	conditional	3/22/95	10
	Onions	6	unconditional	4/1/87	0.06
	Spices	3	unconditional	10/1/90	10
	Vegetables (dried)	3	conditional	3/22/95	10
Russian Federation	Beef (raw, semi-prepared)	3	conditional	7/11/64	8
	Buckwheat mush (dried)	2	unconditional	6/6/66	0.7
	Food concentrates (dried)	2	unconditional	6/6/66	0.7
	Fruit	5	conditional	7/11/64	4
	Fruit (dried)	2	unconditional	2/15/66	1
	Grains	2	unconditional	1/1/59	0.3
	Gruel (dried)	2	unconditional	6/6/66	0.7
	Meat products (prepared)	5	conditional	2/1/67	8
	Onions	6	unconditional	7/17/73	0.06
	Pork (raw, semi-prepared)	5	conditional	7/11/64	8
	Potato	6	unconditional	7/17/73	0.3
	Poultry	5	conditional	7/4/66	6
	Pudding (dried)	2	unconditional	6/6/66	0.7
	Rabbit (raw, semi-prepared)	5	conditional	7/11/64	8
	Rice	2	unconditional	6/6/66	0.7
	Vegetables	5	conditional	7/11/64	4
South Africa (Note: Conditional means that clearances for irradiation of any product are granted to the irradiation facility requesting it.)					
	Almonds (chopped)	3	conditional	5/7/82	10
	Avocado	2	conditional	7/28/77	0.1
	Baby food	3	conditional	5/13/92	10
	Bacon	3	conditional	3/24/89	10
	Beef (dehydrated)	3	conditional	6/8/88	10
	Beef bone extract	3	conditional	1/18/91	20
	Beef soup stock	3	conditional	1/18/91	20
	Bran (raw)	3	conditional	10/14/85	10
	Bread and cake crumbs	3	conditional	11/20/87	10
	Breakfast cereals	3	conditional	4/30/82	8
	Calcium gluconate monohydrate	3	conditional	12/12/90	10
	Casein	3	conditional	9/23/92	10
	Casings (hog)	3	conditional	4/10/91	26
	Cereal drink base	3	conditional	10/16/90	10
	Cereal muesli	3	conditional	2/1/88	10
	Chicken	3	conditional	8/25/78	4
	Coconut (dried)	3	conditional	7/10/90	10
	Cold meats	3	conditional	9/19/89	10
	Condiment paste	3	conditional	5/9/85	10
	Corn flour	3	conditional	11/18/91	10
	Egg (whole, broken)	3	conditional	7/20/89	10
	Egg albumin (powder)	3	conditional	10/12/87	10

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Egg powder	3	conditional	11/20/87	10
	Egg pulp (frozen)	3	conditional	3/29/90	10
	Fish	5	conditional	3/9/87	2
	Frankfurters	3	conditional	9/19/89	10
	Fruit (dried)	3	conditional	10/8/88	10
	Fruit jams	3	conditional	9/3/87	10
	Fruit juices and concentrates	5	conditional	8/11/82	3
	Fruit pulp	3	conditional	9/3/87	5
	Garlic (dried)	3	conditional	7/2/91	10
	Garlic paste	3	conditional	5/24/89	10
	Gelatine	3	conditional	3/5/92	10
	Ginger paste	3	conditional	5/24/89	10
	Green beans	2	conditional	8/11/82	3
	Health drink	3	conditional	4/7/92	10
	Herbs	2	conditional	10/4/85	1
	Honey	3	conditional	10/26/92	13
	Ingredients for health drink mixes	3	conditional	4/7/92	10
	Ingredients for marinades (powder)	3	conditional	7/31/91	10
	Mango	2	conditional	8/25/78	4
	Mango atchar	3	conditional	5/14/87	10
	Meat extract	3	conditional	6/12/85	10
	Milkshake powder	3	conditional	5/2/88	10
	Nuts	3	conditional	4/22/91	10
	Oats (rolled)	3	conditional	4/10/89	10
	Onion powder	3	conditional	11/21/90	10
	Onions	3	conditional	3/19/91	10
	Peanut	3	conditional	10/16/90	10
	Peanut butter	3	conditional	12/12/93	10
	Plum	2	conditional	10/16/90	10
	Pork crackling	3	conditional	7/7/88	10
	Potato	6	conditional	2/6/87	10
	Potato chips (raw, cooked)	3	conditional	8/28/91	10
	Poultry	3	conditional	12/12/89	10
	Rice (brown)	3	conditional	9/20/91	10
	Sausages	3	conditional	9/19/89	10
	Sausages (dried)	3	conditional	4/10/91	10
	Seaweed (dried)	3	conditional	5/25/88	10
	Smoked salami	3	conditional	4/18/91	10
	Sorghum malt beer	5	conditional	3/6/86	1
	Soup powder	3	conditional	10/12/89	10
	Soya fiber	3	conditional	1/23/86	10
	Soya flour	3	conditional	1/23/86	10
	Soya powder	3	conditional	4/25/88	10
	Spices	3	conditional	10/4/85	10
	Starch	3	conditional	1/14/91	10
	Sterile meats	9	conditional	10/1/89	50
	Sugar solutions	3	conditional	12/9/88	10
	Sunflower kernels	2	conditional	2/1/95	10
	Supplements (dietary)	3	conditional	1/29/88	10

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Sweets	3	conditional	8/25/88	10
	Tea, black seed	2	conditional	10/12/93	10
	Tea, comfrey	2	conditional	9/20/92	10
	Tea, rooi bus	2	conditional	1/16/85	10
	Texturized vegetable protein	3	conditional	10/12/93	10
	Tomato	5	conditional	8/11/82	3
	Vegetables (dried)	3	conditional	11/11/83	10
	Viennas	3	conditional	9/19/89	10
	Weight loss preparation	3	conditional	10/11/88	10
	Wors (dried)	3	conditional	6/8/88	10
	Yeast powder (brewers and torulite)	3	conditional	10/25/90	10
Spain	Onions	6	unconditional	9/10/75	0.08
	Potato	6	unconditional	11/4/69	0.15
Syria	Chicken	3	unconditional	8/2/86	7
	Cocoa beans	2	unconditional	8/2/86	5
	Condiments	3	unconditional	8/2/86	10
	Dates	2	unconditional	8/2/86	1
	Fish	5	unconditional	8/2/86	2.2
	Fish (dried)	2	unconditional	8/2/86	1
	Fish products	5	unconditional	8/2/86	2.2
	Legumes	2	unconditional	8/2/86	1
	Mango	2	unconditional	8/2/86	1
	Onions	6	unconditional	8/2/86	0.15
	Papaya	2	unconditional	8/2/86	1
	Potato	6	unconditional	8/2/86	0.15
	Rice	2	unconditional	8/2/86	1
	Spices	3	unconditional	8/2/86	10
	Strawberry	5	unconditional	8/2/86	3
	Wheat	2	unconditional	8/2/86	1
	Wheat products	2	unconditional	8/2/86	1
Thailand	Beans	2	unconditional	12/4/86	1
	Chicken	3, 5	unconditional	12/4/86	7
	Cocoa beans	2	unconditional	12/4/86	1
	Cocoa beans	3	unconditional	12/4/86	5
	Fish	5	unconditional	12/4/86	2
	Fish (dried)	2	unconditional	12/4/86	1
	Fish products	5	unconditional	12/4/86	2
	Garlic	6	unconditional	12/4/86	0.15
	Indian jujubes (dried)	2	unconditional	12/4/86	1
	Mango	1, 2	unconditional	12/4/86	1
	Moo yor (cooked sausage)	3, 7	unconditional	12/4/86	5
	Nham (raw, fermented pork sausage)	3, 7	unconditional	12/4/86	4
	Onions	6	unconditional	12/4/86	0.15
	Papaya	1, 2	unconditional	12/4/86	1
	Potato	6	unconditional	12/4/86	0.15
	Rice	2	unconditional	12/4/86	1
	Sausages	3	unconditional	12/4/86	5
	Shrimp (frozen)	3	unconditional	12/4/86	5
	Spices	2	unconditional	12/4/86	1

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Spices	3	unconditional	12/4/86	10
	Strawberry	5	unconditional	12/4/86	3
	Wheat	2	unconditional	12/4/86	1
	Wheat products	2	unconditional	12/4/86	1
Ukraine	Beef (raw, semi-prepared)	3	conditional	7/11/64	8
	Buckwheat mush (dried)	2	unconditional	6/6/66	0.7
	Food concentrates (dried)	2	unconditional	6/6/66	0.7
	Fruit	5	conditional	7/11/64	4
	Fruit (dried)	2	unconditional	2/15/66	1
	Grains	2	unconditional	1/1/59	0.3
	Gruel (dried)	2	unconditional	6/6/66	0.7
	Meat products (prepared)	5	conditional	2/1/67	8
	Onions	6	unconditional	7/17/73	0.06
	Pork (raw, semi-prepared)	5	conditional	7/11/64	8
	Potato	6	unconditional	7/17/73	0.3
	Poultry	5	conditional	7/4/66	6
	Pudding (dried)	2	unconditional	6/6/66	0.7
	Rabbit (raw, semi-prepared)	5	conditional	7/11/64	8
	Rice	2	unconditional	6/6/66	0.7
	Vegetables	5	conditional	7/11/64	4
United Kingdom (Note: In the UK clearances, "fruit" includes mushrooms, tomatoes, and rhubarb; "vegetables" include pulses; "bulbs and tubers" mean potatoes, yam, onions, shallots, and garlic; "fish and shellfish" includes eels and crustaceans.)					
	Bulbs	6	unconditional	1/1/91	0.2
	Cereal grains	2	unconditional	1/1/91	1
	Condiments	3	unconditional	1/1/91	10
	Fish	5	unconditional	1/1/91	3
	Fruit	2	unconditional	1/1/91	2
	Poultry	3	unconditional	1/1/91	7
	Shellfish	3	unconditional	1/1/91	3
	Spices	3	unconditional	1/1/91	10
	Sterile meals	9	conditional	1/1/91	100
	Vegetable seasonings (dried)	3	unconditional	1/1/91	10
	Vegetables	2	unconditional	1/1/91	1
United States	Enzymes (dehydrated)	3	unconditional	4/18/86	10
	Fruit	2	unconditional	4/18/86	1
	Herbs	3	unconditional	4/18/86	30
	Pork	7	unconditional	7/22/85	1
	Poultry (fresh or frozen)	3	unconditional	9/21/92	3
	Poultry meat (mechanically separated)	3	unconditional	9/21/92	3
	Spices	3	unconditional	4/18/86	30
	Vegetable seasonings (dried)	3	unconditional	4/18/86	30
	Vegetables (fresh)	2	unconditional	4/18/86	1
Uruguay	Potato	6	unconditional	6/23/70	0.15
Vietnam	Fish (dried)	2	conditional	11/3/89	1
	Garlic	6	conditional	11/3/89	0.1
	Green beans	2	conditional	11/3/89	1
	Maize	2	conditional	11/3/89	1
	Onions	6	conditional	11/3/89	0.1

continues

Country	*Item Name*	*Code*	*Type of Clearance*	*Approval Date*	*Dose Max (kGy)*
	Paprika powder	2	conditional	11/3/89	1
	Potato	6	conditional	11/3/89	0.15
Yugoslavia	Cereal grains	2	unconditional	12/17/84	10
	Egg powder	3	unconditional	12/17/84	10
	Fruit (dried)	2	unconditional	12/17/84	10
	Garlic	6	unconditional	12/17/84	10
	Legumes	2	unconditional	12/17/84	10
	Mushrooms (dried)	2	unconditional	12/17/84	10
	Onions	6	unconditional	12/17/84	10
	Potato	6	unconditional	12/17/84	10
	Poultry	3	unconditional	12/17/84	10
	Spices	3	unconditional	12/17/84	10
	Tea extract	3	unconditional	12/17/84	10
	Tea, herbal	3	unconditional	12/17/84	10
	Vegetables (dried)	2	unconditional	12/17/84	10

Explanations for Codes: 1. Delay ripening/physiological growth, **2.** Disinfection, **3.** Microbial control, **4.** Quarantine treatment, **5.** Shelf life extension, **6.** Sprouting inhibition, **7.** Trichina/parasite control, **8.** Sterile meals for hospital patients, **9.** Sterilization, **10.** Unstated.

5 Chill Storage

Rod A. Herbert and Jane P. Sutherland

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5.2 Distribution of psychrophilic and psychrotrophic microorganisms in natural environments
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5.1 INTRODUCTION

Refrigerated storage is now one of the most widely practiced methods of controlling microbial growth in perishable foods. Sales of chilled food products in Europe and North America have increased rapidly in recent years. As a consequence, it is vital that good chill storage procedures are in place to ensure that such foods not only achieve their required shelf lives but are safe for consumption by the end user. While most major food manufacturers and retailers operate a constant and effective cold chain, surveys in the United States (US) have revealed that 20% of domestic and commercial refrigerators operate at temperatures >10°C.[63,138] Because all foodborne pathogens can survive and some can grow at normal refrigeration temperatures (5°–6°C) it is essential, before drawing up legislation governing the refrigerated storage of food products, to understand how microbial growth is affected by low temperatures.

When temperatures are lowered below the optimum for growth of a particular microorganism, lag time and generation time increase and growth rate decreases until, as the temperature approaches the minimum for growth, cell division ceases.[79] At any given temperature, growth rates differ according to the species of organism, and the minimum growth temperature for each organism is different. There can also be considerable strain variation in terms of minimum, maximum, and optimum growth temperatures.

The ability of microorganisms to grow at low temperatures was first demonstrated by Forster in 1887,[37] and subsequently they have been isolated from a diverse range of natural habitats and chill-stored food products.[32,55,91,92,109,120,134] Microbial growth involves an integrated sequence of metabolic steps that are markedly influenced by temperature. The basis of this relationship can be defined mathematically by modifying the equation originally derived by Arrhenius[5] to describe the effects of temperature on chemical reactions:

$$K = Ae^{-E/RT}$$

where K = the velocity constant for a given chemical reaction, R = the gas constant, T = temperature in °K, E = the activation energy, and A = a constant. By substituting growth rate for K and designating E as the temperature characteristic for growth (μ) the equation can be applied to living cells[9]: growth rate = $Ae^{-\mu/RT}$. When a plot of $\log_{10}$ growth rate against 1/°K is made, the slope of the linear portion of the curve equals $-\mu/_{2.303R}$, and from this the temperature characteristic for growth can be determined (Figure 5-1). The linear region of the curve demonstrates the compliance of growth rate with temperature according to the Arrhenius principle. However, the linear region is limited to a restricted temperature range for a given microorganism. Above and below these limits, there is an increasing deviation from linearity until the slope becomes vertical. These deviations from linearity define the temperatures at which growth ceases, and they correspond to the upper and lower limits for growth. Ratkowsky et al.[108] modified the Arrhenius equation and showed that if the square root of the growth rate was plotted against absolute temperature, the relationship was linear with no deviations from linearity between the optimum and lower limits for growth. However, at temperatures approaching the upper limit for growth, the relationship deviated from linearity because of thermal denaturation of cellular proteins. By modifying their original equation Ratkowsky et al.[107] were able to accommodate the deviation from linearity, which they had observed at the upper limit for growth. The revised nonlinear regression model is described by the following equation:

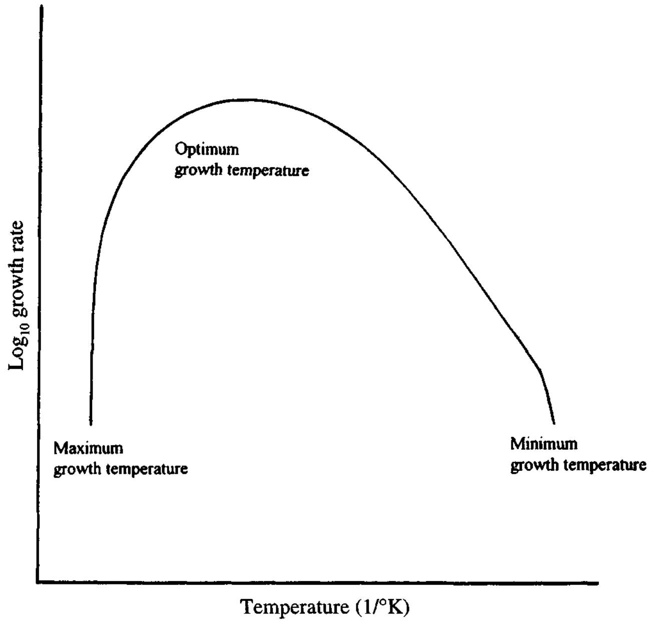

Figure 5-1 Arrhenius Plot Showing the Relationship between Temperature and Growth Rate. *Source:* Copyright © 1989, R.A. Herbert.

$$\sqrt{r} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\}$$

where r = growth rate, T_{min} and T_{max} are the minimum and maximum temperatures at which the growth rate is zero, b = a regression coefficient of the growth rate constant versus degrees Kelvin for temperatures below the optimum for growth, and c = an equivalent regression coefficient for temperatures above the optimum for growth. This model has been extensively tested and shown to apply to all the bacteria investigated.

Microorganisms have characteristic maximum, minimum, and optimum temperatures for growth, the so-called cardinal temperatures.[122] These have been traditionally used by microbiologists to classify microorganisms into various thermal groups according to their ability to grow at low, intermediate, and high temperatures. Microorganisms capable of growing at low temperatures have been termed *psychrophiles,* those at intermediate temperatures *mesophiles,* and those at high temperatures, *thermophiles.* While the latter two groups can be readily distinguished by their growth temperature optima, it is more difficult to precisely define microorganisms that grow at low temperatures. Consequently, there has been extensive debate on the terminology to be used to define those microorganisms that are specifically adapted to grow at low temperatures (psychrophiles) and those that are merely cold tolerant (psychrotrophs).[65,92,93,115,132] Today, the definition proposed by Morita[94] has gained the most widespread acceptance. He defined psychrophiles as microorganisms that have optimum growth temperatures <15°C, a maximum of ~20°C, and a minimum of 0°C or lower; whereas psychrotrophs, while still capable of growth at low temperatures, have temperature optima >15°C and an upper limit for growth >25°C. Psychrophiles have been less frequently isolated than psychrotrophs and tend to be found, although not exclusively, in permanently cold environments.[57,129,134,136] In contrast, psychrotrophs represent a more diverse range of microorganisms, which are readily isolated from environments subject to fluctuating temperature regimes.[120,138]

5.2 DISTRIBUTION OF PSYCHROPHILIC AND PSYCHROTROPHIC MICROORGANISMS IN NATURAL ENVIRONMENTS

Psychrophily and psychrotrophy, the ability to grow at low temperatures, is a common property of many prokaryotes and eukaryotes including eubacteria, cyanobacteria, yeasts, filamentous fungi, and micro-algae.[57,68,92,93,134,141,142] Within the eubacteria, Gram-negative representatives are predominant. For example, Tanner and Herbert showed that in maritime Antarctic waters, where temperatures never exceed 0°C, approximately 50% of the bacteria isolated could be assigned to the genera *Vibrio*/*Aeromonas*, 25% as *Alcaligenes* species, and the remainder to the genera *Pseudomonas* and *Flavobacterium*.[134] Similarly, Kaneko et al.[76] showed that in the Beaufort Sea isolates, identified as *Flavobacterium* species and *Microcyclus* species, were the dominant species present in the surface waters together with *Vibrio* species and other unidentified Gram-negative bacteria. Gram-negative bacteria belonging to the genera *Pseudomonas* and *Alcaligenes* are also dominant in the soils from the maritime Antarctic.[31] In contrast, in the Antarctic Dry valleys of Southern Victoria Land, the soils are dominated by Gram-positive bacteria identified as *Planococcus* species and *Micrococcus* species.[21] In addition to bacteria, a number of psychrophilic yeasts, including *Candida gelida*,[98] *Candida nivalis*,[97] *Leucosporidium scottii*,[149] and *Cryptococcus vishniacii*[142] have also been isolated from Antarctic soils.

Psychrophilic and psychrotrophic microorganisms are not solely confined to the polar regions but have been isolated from a diverse range of temperate environments.[120,130,154] In addition, they have been isolated from dairy products including raw and pasteurized milk,[36] cream,[32] and butter[71]; chilled stored beef[18,51,131]; and vegetables.[60,125] These data indicate that psychrophilic and psychrotrophic microorganisms are ubiquitous in nature and are present in a diverse range of habitats.

The characteristic features of psychrophiles and psychrotrophs are that they are predominantly Gram-negative, aerobic, asporogenous, rod-shaped bacteria. These have been assigned to the genera *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, and *Vibrio*. It is evident that a number of pathogens can grow and may produce toxins at temperatures of 5°C or below. These include *Aeromonas* species, *Clostridium botulinum*, *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterococolitica*, and a number of *Salmonella* species, and this is of particular concern to food microbiologists.

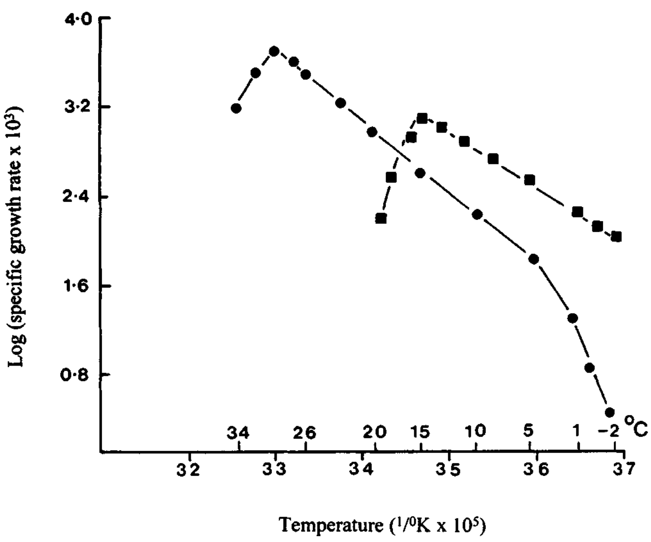

Figure 5-2 Growth Rate and Temperature Relationship. Arrhenius plot showing the relationship between growth rate and temperature for a facultative (●) and an obligate psychrophilic *Pseudomonas* species (■). *Source:* Adapted with permission from W. Harder and H. Veldkamp, Physiology of an Obigately Psychrophilic Marine Pseudomonas Species, *Journal of Applied Bacteriology*, Vol. 31, pp. 12–23, © 1968, Society for Applied Microbiology.

5.3 EFFECT OF TEMPERATURE ON THE GROWTH OF PSYCHROPHILES AND PSYCHROTROPHS

The kinetics of the growth of both psychrophilic and psychrotrophic bacteria have been determined. Data presented in Figure 5-2 show that the difference between these bacteria is in the temperature range over which the Arrhenius curve is linear, rather than the gradient of the linear region of each curve.[52] Thus, the curve for the psychrophilic *Pseudomonas* species does not deviate from linearity until a temperature of –2°C is reached, whereas for the psychrotroph, the slope becomes nonlinear at ~5°C. These data show that psychrophiles have a faster growth rate at low temperatures than psychrotrophs, and this has been confirmed experimentally in competition experiments. Morita and Haight[95] reported that the psychrophile, *Vibrio marinus* MP-1 had a mean generation time of 3.7 hours at 3°C, while Larkin and Stokes[81] claimed that *Bacillus psychrophilus* would grow at –5°C with a doubling time of 6.3 hours. In contrast, psychrotrophic bacteria grow more slowly. For example, *Micrococcus cryophilus* has a mean generation time of 28.2 hours at 0°C, and *Pseudomonas fluorescens* will double its population every 26.41 hours.[58,112] A characteristic feature of psychrophilic microorganisms is their low optimum growth temperature and their inability to tolerate relatively moderate temperatures. Data presented in Table 5-1 show that optimum growth temperatures range from 4°–15°C while maximum growth temperatures, with the exception of *Vibrio*

Table 5-1 Optimum and Maximum Growth Temperatures for a Selected Range of Psychrophiles

Isolate	*Optimal Growth Temperature (°C)*	*Maximum Growth Temperature (°C)*
Vibrio marinus MP-1	15	20
Ant-300 (*Vibrio* species)	7	13
Pseudomonas species	14	19
Vibrio AF-1	15	21
Vibrio AM-1	6	16
Vibrio BM-2	4	14
Vibrio BM-4	8	17

Source: Copyright © 1989, R.A. Herbert.

species Ant-300, are in the range 18°–20°C. While growth of microorganisms has been reported at temperatures below 0°C, there is currently no consensus of what temperature constitutes the lower limit for growth. Theoretically, the lowest temperature that might allow growth to occur is ~ –130°C, since below this temperature no "free" water is available.[151] In practice, the lowest temperature at which microbial growth has been unequivocally demonstrated is –12°C for bacteria,[66] although in the literature there are unsubstantiated reports of fungi growing at –20°C and yeasts at –34°C. These finding are consistent with the observation that at temperatures < –10° to –15°C, intracellular water begins to freeze resulting in ionic imbalance, pH shifts, and a concomitant reduction in water activity. These changes, either singly or in combination, exert a toxic effect on the cells that is either lethal or severely impairs metabolic function.[115] Thus, the minimum growth temperature of psychrophiles is set by the temperature at which their cytosol freezes. However, at temperatures above the minimum for growth and where ice is present, growth is also influenced by water activity (a_w). Thus, in part-frozen systems, the a_w is reduced, for example at –5°C the a_w is lowered to ~0.95, while at –10°C the value is ~0.9. Microorganisms, growing under such conditions must therefore be not only cold tolerant but also able to grow at low water activities.

5.4 EFFECTS OF TEMPERATURE ON SOLUTE UPTAKE

From the preceding section, it is evident that psychrophiles form a distinct thermal group because of their ability to grow rapidly at low temperatures. To achieve rapid growth, it is essential that substrate uptake is not the limiting step. A number of workers have proposed that the minimum temperature for growth of psychrotrophic microorganisms is governed by their capacity to transport solute molecules across the cytoplasmic membrane.[38,57,67,94] Support for this hypothesis has come from comparative studies on the respiratory activity of a psychrotrophic *Candida* species and a mesophilic strain of *Candida lipolytica* grown at different temperatures.[9] The psychrotroph was able to respire exogenous glucose at 0°C, whereas the mesophile showed no activity at temperatures <5°C. However, the mesophile was able to respire endogenous substrates at temperatures <5°C, implying that the limiting factor was the ability to transport sugars into the cell at near-zero temperatures. Similar results have also been reported for mesophilic strains of *Candida utilis* and *Arthrobacter* species.[100] While psychrotrophs continue to transport substrates at low temperatures (~0°C), the rates recorded are significantly lower than those measured at higher temperatures, thus confirming that they are cold tolerant rather than cold-adapted organisms. This was demonstrated by Herbert and Bell[57] in their comparative study of glucose and lactose uptake by psychrophilic and psychrotrophic bacteria. These workers showed that sugar uptake in the psychrophilic bacteria was maximal at 0°C and decreased with increasing temperature, whereas the converse was observed with the psychrotrophs. These results are in agreement with those obtained by Fukunaga and Russell.[38] However, not all psychrophilic microorganisms show this pattern of substrate uptake. For example, in *Micrococcus cryophilus,* substrate uptake was, within the limits of experimental error, the same at 0°C and 20°C.[112] Similarly, uptake of L-leucine and 2-deoxyglucose by a psychrophilic Gram-positive bacterium remained unaffected during growth at low temperatures.[152]

It is clearly evident that psychrophiles/psychrotrophs can be distinguished from mesophiles by their ability to transport solutes at temperatures <5°C. Farrell and Rose[34] proposed a number of mechanisms to account for the inability of mesophiles to transport solutes at near-zero temperatures. These include:

1. Solute carrier proteins in the cytoplasmic membrane are less sensitive to low temperature inactivation than those in mesophiles.
2. Solute carrier proteins in mesophilic microorganisms are not abnormally cold labile, but owing to changes in the molecular architecture of the lipid bilayer at low temperatures, they are unable to bind to their respective substrates.

3. At low temperatures, there is insufficient energy available to energize membrane transport systems in mesophiles.

To date, there are no convincing data to show that individual solute transport proteins in mesophiles are abnormally cold sensitive or that ATP availability limits solute uptake. As a result, the majority of studies have focused on determining the effects of low temperatures on the lipid composition of the cytoplasmic membrane and how these changes modify the liquid-crystalline state of the lipid bilayer.

5.5 EFFECTS OF TEMPERATURE ON MEMBRANE STRUCTURE AND FUNCTION

5.5.1 Modification of Membrane Composition

Lipids are a major component of all cell membranes and constitute some 40% to 70% of the total membrane dry weight.[56] As in higher organisms, glycerol phosphatides are the predominant structural lipid class present in bacterial cell membranes and are fatty acid esters of sn-glycerol-3-phosphate. Phosphatidylethanolamine, the most widely distributed phospholipid in bacterial cytoplasmic membranes, is often the dominant component in Gram-negative species. In addition, phosphatidylglycerol and diphosphatidylglycerol (cardiolipin) are commonly found in bacteria, whereas diphosphatidylserine is normally a minor component because it is rapidly decarboxylated to give phosphatidylethanolamine. While phosphatidylinositol and phosphatidylglycerol are important components of eukaryote membrane systems, they are uncommon in bacteria.[46] Equally, bacterial membranes rarely contain sterols such as cholesterol and, if present, the quantities synthesized are insignificant compared with those found in higher organisms. Their absence in prokaryote membrane systems has proved extremely useful when investigating the effects of temperature on gel to liquid-crystalline phase transitions in lipid bilayers.

Within the cell membrane, the phospholipid molecules are arranged in the form of a bilayer with the polar head groups exposed at the extracellular and intracellular surfaces. As a consequence these groups are able to interact with the aqueous environment on the inside and outside of the cell. The hydrophobic fatty acid acyl chains, in contrast, are stacked in a parallel manner perpendicular to the plane of the membrane, with the terminal methyl groups located deep in the interior of the bilayer. Fatty acids in bacterial cells membranes are typically 10 to 20 carbon atoms in length, with C_{16} to C_{18} typically predominating. Bacterial fatty acids may be either straight chain saturated, straight chain unsaturated, branched chain (iso or anteiso), or cyclopropane acids. Long chain polyunsaturated fatty acids, commonly found in eukaryotes, are normally absent in bacteria. Cyclopropane fatty acids are, however, widely distributed among bacteria, while iso and anteiso branched chain acids predominate among the genera *Micrococcus* and *Bacillus.*

Modification of membrane lipid composition in response to lowering of the growth temperature usually involves changes in the hydrophobic acyl chains rather than the polar head groups.[113] This is because the changes in fatty acid composition are more effective in modifying and maintaining membrane fluidity than changes in head group composition. It has been proposed that the physiological significance of these changes in lipid composition is to maintain the lipid bilayer in the fluid liquid-crystalline state.[124] This process, termed *homeoviscous adaptation,* implies that membrane fluidity is maintained at a relatively constant level irrespective of growth temperature. However, in practice, a constant level of membrane fluidity is rarely achieved and under such conditions all that may be required is that there is sufficient membrane fluidity within the lipid bilayer to maintain the liquid-crystalline state. Such a condition might therefore be better described as *homeophasic adaptation.*[87,122]

The most commonly observed response to a lowering of growth temperature is an increase in the proportion of unsaturated fatty acids at the expense of the corresponding saturated acids. This has been elegantly demonstrated in a comparative study of psychrophilic and mesophilic yeasts by Brown and Rose,[20] who showed that at low temperatures the psychrophiles synthesized increased levels of hexadecenoic (16:1) and octadecenoic (18:1) fatty acids compared with their mesophilic counterparts. While similar changes have been recorded in bacteria, the response to low temperatures is considerably more complex and, in addition to changes in the degree of unsaturation of membrane lipids, other modifications may occur, eg, an increase in the proportion of branched chain fatty acids, shortening of the acyl chain length, or a reduction in the proportion of cyclopropane fatty acids.[114,115] The net effect of these changes is to produce membrane lipids with a lowered gel to liquid-crystalline state transition temperature, thereby maintaining membrane function at low temperatures. Data presented in Table 5-2 show that a psychrotrophic strain of *Pseudomonas fluorescens* responded to growth at low temperatures by synthesizing increasing proportions of hexadecenoic and octadecenoic acids while synthesis of the cyclopropane heptadecanoic acid was inhibited at temperatures of 10°C and below. In *Vibrio* AM-1, isolated from the maritime Antarctic, the response to a decrease in growth temperature was to synthesize increased quantities of hexadecenoic (C16:1) and heptadecenoic acid (C17:1) (Table 5-2).[13] A different response was observed in *Vibrio* BM-2, which synthesised increased proportions of short chain fatty acids (C9:1, C12:1, and C14:1) at low temperatures, while the levels of hexadecenoic and heptadecenoic acids showed little change. This response is similar to that recorded by Russell[112] for *M. cryophilus* when grown at low temperatures. The physiological effect of acyl chain shortening is considered to be the same as increasing the proportion of unsaturated fatty acids in the membrane lipids, ie, to maintain a mobile hydrophobic core at low temperatures.

While many microorganisms have been shown to modify their membrane lipid composition in response to a decrease in growth temperature, it is not an absolute requirement; there are several reports in the literature of organisms that show little change in fatty acid composition over a wide temperature range. For example, Hunter and Rose[62] were unable to demonstrate any significant difference in the fatty acid composition of *Saccharomyces cerevisiae* when grown at 15° and 30°C. Similarly, Bhakoo and Herbert[14] were unable to demonstrate significant differences in the lipid composition of psychrotrophic *Pseudomonas* species isolated from maritime Antarctic waters (strain T-6, Table 5-2). In *Pseudomonas* T-6 hexadecenoic (C16:1) and octadecenoic acid (C18:1) were the dominant fatty acids present in the membrane lipids and showed no significant change over the temperature range 0°–20°C (Table 5-3). These data question the absolute need for all prokaryotes to modify their membrane lipid composition as a physiological requirement for growth at low ambient temperatures. Experiments with fatty acid auxotrophs of *E. coli* and *Acholeplasma laidlawii*, in which the membrane lipid composition was varied in a controlled manner to provide a wide range of membrane fluidities, demonstrated that both bacteria continued to grow even though there were major differences in fluidity within the liquid-crytalline phase. Both bacteria could tolerate substantial proportions of gel phase lipid in their cyto-

Table 5-2 Effect of Growth Temperature on the Fatty Acid Composition (expressed as the percentage by weight of the total fatty acids) of Some Gram-Negative Bacteria

	Pseudomonas fluorescens† *for Growth Temperatures of*			Pseudomonas *T-6‡ for Growth Temperatures of*			Vibrio *AM-1§ for Growth Temperatures of*			Vibrio *BM-2§ for Growth Temperatures of*		
*Fatty acid**	*10°C*	*20°C*	*30°C*	*0°C*	*8°C*	*20°C*	*0°C*	*8°C*	*15°C*	*0°C*	*8°C*	*15°C*
9:1	—	—	—	—	—	—	—	—	—	11.4	6.6	5.4
12:0	—	—	—	1.5	2.3	2.1	—	—	0.8	0	6.4	9.0
12:1	—	—	—	—	—	—	8.2	10.7	1.0	13.7	1.0	—
13:0	—	—	—	2.2	2.6	—	—	—	—	—	—	—
13:1	—	—	—	4.4	8.1	9.9	—	—	—	—	—	—
14:0	—	trace	trace	5.1	6.1	9.9	1.2	1.7	21.8	—	1.0	1.2
14:1	—	—	—	4.3	1.7	—	5.5	—	—	20.6	7.5	12.0
15:0	—	—	—	—	—	—	—	—	—	0.7	0.9	1.1
15:1	—	—	—	1.1	1.2	—	8.2	1.0	18.2	1.5	2.5	2.9
16:0	20.4	29.5	34.4	1.1	1.6	1.4	5.1	8.2	17.8	21.6	46.5	38.5
16:1	52.7	46.2	30.9	56.6	54.5	59.0	38.6	39.6	—	20.3	17.7	22.0
17:0	—	—	—	—	—	—	8.6	11.1	13.5	0.4	0.4	0.4
17:0Δ	—	5.7	18.1	—	—	—	—	—	—	—	—	—
17:1	—	—	—	7.1	5.3	2.4	20.8	21.9	—	1.3	1.4	1.2
18:0	—	—	—	0.4	1.0	1.3	—	—	—	—	—	—
18:1	26.9	18.6	16.2	15.9	15.1	14.2	—	—	—	—	—	—

*In fatty acid designations, the first number indicates the number of carbon atoms and the second the number of double bonds; Δ indicates a cyclopropane ring.

†Data from Gill.[42]

‡Data from Bhakoo and Herbert.[13]

§Data from Bhakoo and Herbert.[14]

plasmic membranes without loss of membrane function, viz. up to 50% for *E. coli*[70] and 80% to 90% for *A. laidlawii*.[88] These results indicate that there are clear upper and lower limits of membrane fluidity for growth. However, they give no indication of the optimum membrane fluidity. In *E. coli* grown at 37°C, the minimum requirement for unsaturated fatty acids for growth was approximately 20% of the total fatty acid composition.[25] Analysis of the fatty acid profiles of the psychrotrophic pseudomonads and psychrophilic *Vibrio* species show that in all these bacteria the levels of unsaturated fatty acids are considerably in excess of the minimum required for growth. These data support the theory proposed by McElhaney[87] that maintenance of the correct lipid phase is more important than conservation of some absolute value of membrane fluidity.

Equally equivocal are the patterns of change in membrane phospholipids in response to temperature. In a comprehensive study, Bhakoo and Herbert[13,14] investigated the effects of temperature on the membrane phospholipid composition of four psychrophilic *Vibrio* species and four psychrotrophic *Pseudomonas* species. In the psychrophiles, there was a 43% increase in total phospholipid content at 0°C compared with that at 15°C (Table 5-3), accompanied by an increase in cell size.[57] In contrast, in *Pseudomonas* T-6 the phospholipid content remained relatively constant over the temperature range 0°–20°C. Similar results have been reported for *Pseudomonas fluorescens*[42] and other marine psychrotrophic pseudomonads.[19] In *Vibrio* BM-4, diphosphatidylglycerol synthetase and phosphatidylethanolamine decarboxylase were abnormally temperature sensitive and inactivated at 15°C.[12] This finding explains the sharp decline in phosphatidylethanolamine and diphosphatidylglycerol levels recorded at 15°C and the concomitant accumulation of their immediate precursors. In contrast, the same enzymes in the psychrotrophic pseudomonads were thermostable.

5.5.2 Biochemical Basis of Changes in Membrane Fatty Acid Composition at Low Temperatures

While there is an extensive body of data to show the effects of temperature on lipid composition, the mechanisms by which these changes are effected are less well understood. A major difficulty is that these modification processes are regulated in a complex manner at different levels. For example, it is difficult to determine whether temperature directly affects the enzymes involved or whether the effect is an indirect one mediated via membrane fluidity.[115] Clarke[23] summarized the following different levels at which regulation of membrane lipid occurs:

1. regulation of transcription by *de novo* synthesis of desaturase enzymes
2. regulation at the level of enzyme activity by temperature-induced modifications of desaturase enzymes
3. regulation at the level of the membrane by modulating the activity and/or specificity of the glycerophosphateacyltransferase system
4. regulation by control of the relative balance of synthesis and desaturation of fatty acids available to the membranes

In a series of elegant experiments, Fulco[39,40] showed that in *Bacillus megaterium* temperature acted directly on the desaturase by controlling its turnover rate and the transcription of a temperature-sensitive modulator molecule. In this bacterium the desaturase, which only desaturates at the Δ^5 position, is absent in cultures grown at 35°C, whereas at 20°C the enzyme is synthesized. Addition of

Table 5-3 Effect of Temperature on the Phospholipid Composition (μg phospholipid mg^{-1} dry weight) of the Psychrophile *Vibrio* BM-4 and the Psychrotroph *Pseudomonas* T-6 Grown in Continuous Culture under Glucose Limitation

	Vibrio *BM-4**			Pseudomonas *T-6*†		
Phospholipid	*0°C*	*8°C*	*15°C*	*0°C*	*8°C*	*20°C*
Phosphatidylserine	4.5	7.0	10.6	7.2	6.2	5.8
Phosphatidylglycerol	53.3	22.0	55.9	18.5	20.6	22.4
Phosphatidylethanolamine	25.6	21.2	13.3	112.2	114.2	94.2
Diphosphatidylglycerol	86.6	80.0	38.7	39.6	38.6	32.6
Total phospholipids	169.4	131.0	118.5	177.5	179.6	155.2

*Data from Bhakoo and Herbert.[13]
†Data from Bhakoo and Herbert.[14]

chloramphenicol to cell suspensions showed that enzyme synthesis was *de novo*. Similar responses have been reported for *Pseudomonas* E-3 and the Gram-negative bacterium SCRC-2738 when grown at low temperatures.[2,144]

In *Escherichia coli*, the ratio of saturated to unsaturated fatty acids incorporated into membrane lipids is regulated by the relative activities of β-ketoacyl-ACP synthetases, which exist in two forms: synthetase I and synthetase II. In this bacterium, the fatty acid synthetases produce both saturated and unsaturated fatty acids as a result of a branch in the biosynthetic pathway at β-OH-decanoyl-ACP. This C_{10} fatty acid intermediate gives rise to either palmitic (16:0) or palmitoleic acid (16:1 Δ^9 cis). β-ketoacyl-ACP synthetase II is more efficient in elongating palmitoleic acid to cis-vaccenic acid (18:1) and is relatively more temperature sensitive than synthase I. As a consequence, a greater proportion of cis-vaccenic acid is synthesized at low temperatures.[143] Fatty acid composition in *E. coli* is therefore mediated by temperature modulation of preformed enzymes.

The glycerol phosphate acyltransferase system has been proposed to play an important role in controlling the quantity and type of fatty acids acylated into phospholipids.[116] This enzyme exists in an active monomeric and inactive oligomeric form, and it has been postulated that reversible oligomerization is the mechanism whereby the activity of this enzyme system is regulated.[117] A number of studies have shown that the glycerol phosphate acyltransferase system is sensitive to lipid acyl composition, and it has been proposed that this enzyme functions by "sensing" changes in membrane fluidity as a result of temperature shifts.[88] However, the experimental evidence to support this hypothesis is still lacking.

It has been demonstrated that many microorganisms respond to a rapid temperature downshift by aerobic desaturation of preexisting saturated fatty acids.[113] Most workers consider the substrate to be membrane lipid, and this system provides a rapid initial modification of existing membrane lipid in response to sudden temperature changes. The advantage of this system is that it enables rapid modification in fatty acid composition of existing membrane lipid for the minimum expenditure of energy in response to temperature change.

5.6 EFFECT OF TEMPERATURE ON PROTEIN SYNTHESIS AND ENZYME ACTIVITY

Because psychrophiles are able to grow at low temperatures, it is implicit that their enzymes are able to maintain structural integrity and catalytic function at these temperatures. However, in contrast to the extensive literature on mesophilic and thermophilic enzymes, there is a paucity of data on how the intrinsic structure of psychrophilic proteins enables them to function efficiently at low temperatures. Two experimental approaches have been adopted to investigate the mechanisms of thermostability of proteins at different temperatures: (1) determination of the amino acid sequence of the native protein and site-directed mutant forms of the same enzyme but with altered thermostability, and (2) comparative studies of the same enzyme isolated from psychrophilic, mesophilic, and thermophilic microorganisms. Results from these different experimental approaches have yielded essentially the same conclusion, namely that the thermal stability of proteins is an intrinsic property of the peptide chain.[84] Schlatter et al.[118] determined the amino acid sequence of lactate dehydrogenase obtained from *Bacillus psychrosaccharolyticus*. The enzyme from this psychrotrophic bacterium has a significantly lower temperature optimum than the equivalent enzyme isolated from closely related mesophilic and thermophilic *Bacillus* species. It has been postulated that the psychrophilic enzyme should have weaker hydrophobic bonding in the core region and additional hydrophobic bonds at the surface.[158] However, experimental evidence to support this hypothesis is lacking and must await the results from comparative studies in order to identify the specific amino acid substitutions that confer psychrophilic properties on the enzyme. The search for key amino acid substitutions is complicated by the fact that free energy of the native and denatured state of an enzyme is small.[85] As a consequence only a few amino acid residues may be involved.

Recently, Ochiai et al.[101] demonstrated that the psychrophilic bacterium, *Vibrio* ABE-1 synthesizes two isocitrate dehydrogenase isoenzymes that exhibit different thermal characteristics. The monomeric form, isoenzyme I, appears to be cold adapted and functions at 0°C but is denatured at temperatures >15°C. In contrast, the dimeric form, isoenzyme II, is more thermostable but exhibits decreased activity at low temperatures. The two isoenzymes have a similar amino acid composition although there are slight differences in the content of nonpolar and hydroxyl amino acids.[69] Whether these differences in amino acid composition are sufficient to account for the observed differences in thermostability and activity has yet to be demonstrated.

A further feature of microbial growth at low temperatures is that protein synthesis must also function at such temperatures. Several comparative studies of *in vitro* protein synthesis in mesophiles and psychrophiles have demonstrated that in the latter organisms the miscoding rate at 0°C was lower.[80] This study indicated that the ability to synthesize proteins efficiently at 0°C was an intrinsic property of the ribosomes and not of soluble components in the cell-free extract. Szer[133] isolated a ribosomal protein(s) from the psychrophilic bacterium, *Pseudomonas* strain 412, which confers the ability to translate specific mRNAs at 0°C. When this protein(s) was removed by washing, the ribosomes lost the capacity to synthesise proteins at low temperature. Araki[4] compared rates of protein synthesis in cell-free extracts prepared from *Vibrio* ANT-300 at 0° and 13°C and showed that the process was regulated by soluble factors. He identified 24 soluble proteins whose concentrations changed with temperature but no functions have been ascribed to any of them.

In natural environments where microorganisms are commonly subject to rapid changes in temperature, it is perhaps not surprising that they have evolved protection mechanisms. *E. coli*, for example, synthesizes cold-shock proteins in response to a temperature downshift.[73] Several of these cold-shock proteins have been identified and appear to function mainly in translation, although one major protein (CS 7.4) has been proposed to function, due to its small size, as an antifreeze protein.[73] The psychrotrophic yeast, *Trichosporon pullulans* synthesizes up to 26 different cold-shock proteins in response to a temperature downshift.[74] These data indicate that induction of cold-shock proteins enables microorganisms to withstand and survive rapid changes in growth temperature and must be taken into consideration when determining protocols for the refrigerated storage of food products.

5.7 GROWTH AND SURVIVAL OF FOOD-ASSOCIATED MICROORGANISMS AT LOW TEMPERATURES

5.7.1 Growth at Low Temperatures

Data presented in Table 5-4 show the minimum, maximum, and optimum temperatures for the growth of foodborne pathogens. It is evident from

Table 5-4 Minimum, Maximum, and Optimum Temperatures for Growth of Foodborne Pathogenic Microorganisms

Organism	T_{min} (°C)	T_{opt} (°C)	T_{max} (°C)	*Reference*
A. hydrophila	0–4	28–35	42–45	64
B. cereus				
psychrotrophic	4–5[a]	28–35[b]	30–35[a]	[a]64, [b]41
mesophilic	10–15[a]	35–40[b]	50–55[a]	[a]64, [b]71
Campylobacter	32	42–45	45	29
Cl. botulinum				
proteolytic	12.5	35	50	105
nonproteolytic*	3.0–3.3[a,b]	30[b]	45[b]	[a]49, [b]105
Cl. perfringens	12	43–47	50	64
E. coli (pathogenic)†	7	35–40	46	64
L. monocytogenes	0–4[a,b]	30–37	45	[a]45, [b]145
Pl. shigelloides	8–10[a,b]	30	45	[a]119, [b]90
Salmonellae	5	35–37	45–47	123
Shigella‡	7	37	45–47	35
Staph. aureus§	7	35–40	48	135
V. cholerae	10	37	43	64
V. parahaemolyticus	5[a]	37[b]	43	[a]10, [b]137
Y. enterocolitica	–1[a]	28–30[b]	42[c]	[a]146, [b]140, [c]54

Notes: T_{min} minimum growth temperature.
T_{opt} optimum growth temperature.
T_{max} maximum growth temperature.

*Nonproteolytic *C. botulinum* also forms toxin at low temperatures. See Chapter 41 for additional details.

†For *E. coli* 0157:H7, the range is slightly more limited, minimum growth temperature being 8°C and maximum 42°–44°C.

‡These values apply to *S. sonnei*. The growth range for *S. flexneri* is more restricted, ie, from 10°–44°C.

§For enterotoxin production, the range is 10°–46°C and the optimum is 40°–45°C.[137]

these data that a number of pathogens can not only grow but also may produce toxin(s) at temperatures of 5°C or below. Consequently, growth can take place under commercial and domestic refrigeration conditions by organisms such as *Aeromonas* species, psychrotrophic *Bacillus cereus*, nonproteolytic *Clostridium botulinum, Listeria monocytogenes,* and *Yersinia enterocolitica*. Some strains of salmonellae, such as *S. bredeny, S. virchow,* and *S. typhimurium* can grow at 5°–6°C, and *S. agona, S. senftenberg,* and *S. hadar* grow at temperatures just above 6°C.[3] While growth at such temperatures may be relatively slow, the potential exists for unacceptable multiplication to occur, given the commercial requirement for long shelf lives to allow for storage at the factory prior to distribution, the distribution process itself, subsequent display in shops and supermarkets, and finally, customer storage prior to consumption.

It is evident from the data presented in Table 5-5 that while the growth of pathogens such as nonproteolytic *Clostridium botulinum* is very slow in nutrient medium at 4°C, a relatively modest temperature increase to 8°C leads to an almost five-fold increase in growth rate. Similarly, psychrotrophic *Bacillus cereus* grows slowly at 5°C but at 7°C the growth rate is almost double. These data demonstrate how a slight increase in temperature (2°–4°C) can not only result in the rapid growth of pathogens but also can create the potential for toxin produc-

Table 5-5 Effect of Temperature on Generation Time of Pathogenic Microorganisms Growing in Media

Organism	*Substrate*	*Temperature (°C)*	*NaCl (% w/v)*	*pH*	*Generation Time (Hours)*	*Source of Data*
A. hydrophila	nutrient broth	3	0.5	7.0	9.3	86
		5	0.5	7.0	3.7	
		10	0.5	7.0	2.2	
B. cereus (psychrotrophic)	tryptone soya broth	5	0	7.0	22.0	Walker et al. (unpubl.)*
		7	0	7.0	13.4	
		10	0.5	7.0	12.1	
Cl. botulinum (nonproteolytic)	peptone-yeast-glucose-starch (phosphate buffered)	4	<0.1	6.7	42.0	48
		8	<0.1	6.7	9.0	
		10	<0.1	6.7	5.7	
		15	<0.1	6.7	2.4	
E. coli 0157:H7 strain 30-2C4	tryptone soya broth	8	0.5	6.8	12.7	Sutherland et al. (unpubl.)†
E. coli 0157:H7 (4-strain combination)	tryptone soya broth	10	0.5	7.0	7.4	Sutherland et al. (unpubl.)†
		15	0.5	7.0	2.8	
L. monocytogenes	tryptone soya broth	3	0.5	7.0	25.3	McClure et al. (unpubl.)‡
		5	0.5	7.4	17.6	
		10	0.5	7.4	5.2	
Salmonellae	tryptone soya broth	8	0.5	7.1	15.9	Sutherland et al. (unpubl.)†
		10	0.8	6.2	13.7	
		15	0.8	6.2	2.4	
Salmonella heidelberg	tryptone soya broth	6.7	0.5	7.3	29.3	84
		7.5	0.5	7.3	22.8	
		8.3	0.5	7.3	17.9	
Staph. aureus	brain heart infusion	8	0.5	6.0	15.1	Sutherland et al. (unpubl.)†
		10	0.5	6.5	12.0	
		15	0.5	6.0	4.0	
Y. enterocolitica	brain heart infusion	5	0.5	7.1	9.2	Sutherland et al. (unpubl.)†
		10	0.5	7.0	3.8	
		15	0.5	6.1	2.2	

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‡ Unpublished data of McClure, P.J., et al., used in preparation of Food MicroModel. Reproduced by permission of the Ministry of Agriculture, Fisheries and Food, UK.

tion. Of particular concern to food microbiologists is the fact that refrigeration at temperatures above 3°C cannot be regarded as an absolute safeguard against growth of and toxin production by nonproteolytic *Clostridium botulinum.*[49] Data presented in Table 5-6 show that the growth of pathogens in a diverse range of food products can be rapid at currently permitted refrigeration temperatures (5°–8°C). Growth of food spoilage microorganisms as opposed to pathogens can be even more rapid at these temperatures (Table 5-7). Thus, *Pseudomonas* species growing on meat slices stored at 2°C have mean generation times of 7.6 to 8.2 hours. Similarly, *Brochothrix thermosphacta* growing on bologna sausage stored at 2°C has a generation time of 8.2 hours. Data presented in Table 5-7 illustrate how a relatively modest increase in storage temperature from 2° to 5°C can lead to a significant increase in growth rate of fluorescent pseudomonads with a concomitant reduction in shelf life of the food product. These data graphically demonstrate the growth potential of psychrotrophic and psychrophilic spoilage microor-

Table 5-6 Effect of Temperature on Generation Time of Selected Pathogenic Bacteria Growing in Food

Organism	*Temperature (°C)*	*Food Product*	*Generation Time (hours)*	*Reference*
A. hydrophila	5	ground raw pork	13.9	102
	5	raw lamb pieces	36.5	28
	10	cooked pork	8.4	129
	10	cold-smoked salmon	9.4	61
B. cereus	5	liver sausage	8.3	6
	5	reconstituted milk	41, 69	109
	7	skimmed milk	10–63	30
	15	cooked rice	3–4.3	72
Cl. perfringens	12	frankfurters	11.5	127
	15	frankfurters	6.5	
	15	cooked ground beef	44.2	75
E. coli (pathogenic)	8	high pH beef	9.0	43
	10		5.4	
	12		5.4	
	15		3.7	
E. coli 0157:H7	8	milk	20.6	147
	15		4.6	
L. monocytogenes	4		30–33	111
	10		7–10	83
	4	raw beef/pork mix	22.8	77
	8		9.6	
	12		6.0	
Salmonellae	8	sterile beef	35.0	82
	10		9.4	
	12		4.6	
	10	egg albumen	11.1	7
	20		16.1	
	20	egg yolk	5.2	
	10	tofu	12.7	78
	15		5.7	
Staph. aureus	8	bologna sausage	37.0	100
	10		25.3	
	12		6.8	
	10	imitation crab	12.5	155
	15		4.7	
	10	tofu	19.0	78
	15		6.1	

Table 5-7 Effect of Temperature on Generation Time of Food Spoilage Microorganisms Growing in Food

Organism	*Temperature (°C)*	*Substrate*	*Generation Time (hours)*	*Reference*
Pseudomonas species	2	meat slices	7.6	44
	5		5.1	
	10		2.8	
	15		2.0	
	4	beef muscle	9.0	27
	25		0.9	
	0	milk	26.6	50
	5		11.7	
	10		5.4	
Pseudomonas species (fluorescent)	2	meat slices	8.2	44
	5		5.4	
	10		3.0	
	15		2.0	
	4	beef muscle	9.6	27
	25		2.2	
	0	milk	29.1	50
	5		14.7	
	10		6.52	
Brochothrix thermosphacta	2	meat slices	12.0	44
	5		7.3	
	10		3.4	
	15		2.8	
	2	bologna sausage	8.2	99
	5		4.4	
	10		2.9	
	4	sausage meat	6.6	8
	10		3.3	
Lactobacillus plantarum	8	minced lamb	8.8	Curtis and Petitt (unpubl.)*
	25		1.6	
	8	beef steak (vacuum packed)	8.8	
	25		1.3	
	8	UHT fat-free milk	1.6	
	25		11.2	

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ganisms at temperatures that have, until relatively recently, been considered adequate for the refrigerated storage of perishable food products.

5.7.2 Survival of Pathogens at Low Temperatures

It is paradoxical that while refrigeration prolongs the shelf life of perishable foods and prevents the growth of some organisms, it also promotes the survival of foodborne pathogens unable to grow at low temperatures (Table 5-8). For example, the survival of verocytotoxigenic *Escherichia coli* in acidic environments such as apple juice[88] and mayonnaise[53,106,150,156] is appreciably longer at 5°–7°C than at 25°C.

It has been suggested that virulence of pathogenic bacteria can be influenced by the temperature of growth, but the relevant evidence is conflicting. Growth at low temperature was reported to increase virulence of *L. monocytogenes* when assessed by intravenous injection into animals, but not when the

Table 5-8 Effect of Temperature on Death Rate of Pathogenic Microorganisms Surviving in Food

Organism	*Temperature (°C)*	*Food Product*	*T_{1000} (days)*	*T_{100} (days)*	*T_{10} (days)*	*Reference*
Campylobacter	4	turkey roll in air	2.21			104
	21		0.82			
	4	turkey roll/MAP*	9.0			
	21		1.55			
	1	raw beef	9–20			22
	20		2.8–4.0			
	10	reconstituted skimmed milk	8.0–10.0			
	20		0.8–1.5			
	4	cooked chicken breast	23–55			15
	23		11–20			
E. coli O157:H7	8	apple cider (fermented)	9.5–29			157
	25		2.6–4.8			
	7	mayonnaise	19–26			150
	25		0.5–0.7			
	21	roast beef salad			0.5–0.63	1
	30		</=0.25			
	21	roast beef slurries			0.75–0.83	
	30		</=0.38			
L. monocytogenes	4	salted skimmed milk	550–975			103
	22		70–390			
	4	salted whey	557–975			
	22		80–390			
	4	macaroni salad with	75			33
	12.8	mayonnaise	7.5			
	4	macaroni salad with	150			
	12.8	reduced calorie mayonnaise	5.0			
Salmonellae	5	pasteurized apple juice		2.3		36
	22		<0.1			
	4	pepperoni sausage		25–38		126
	35				1.0–1.3	
	4	chocolate egg liqueur	6.7			148
	22		2.3			

Notes:

T_{1000} = time to achieve a 3-log reduction in number.
T_{100} = time to achieve a 2-log reduction in number.
T_{10} = time to achieve a 1-log reduction in number.

*MAP, modified atmosphere packaging.

bacteria were administered intragastrically.[26] However, in a later study, growth at low temperature showed no effect on virulence when the organisms were administered by either route.[96]

In some strains of *Y. enterocolitica*, growth at low temperature and suboptimal pH has been reported to increase loss of the virulence plasmid.[16] No change in the fatty acid composition of the cell membrane resulted from growth at low temperature, so this factor was not responsible for the increase in loss of the virulence plasmid.

5.7.3 Factors Influencing Growth and Survival at Low Temperatures

Minimum growth temperatures for microorganisms are influenced by many factors and are lowest when other growth-influencing factors are optimal. Thus, the data presented in Table 5-4 regarding minimum, maximum, and optimum temperatures assume optimal conditions for growth, ie, the substrate was microbiological medium, the pH value was close to 7.0, there was no added humectant to reduce the water activity, and no preservatives present.

Comparison of generation times in Table 5-5 with those of relevant organisms in Table 5-9 shows that the further the environmental conditions deviate from the optimum for a given organism, the greater the corresponding decrease in growth rate. Some bacteria can be highly resistant to specific environmental conditions. For example, the growth rates of *Staphylococcus aureus* and psychrotrophic *Bacillus cereus* are relatively unaffected, even at temperatures near the minimum for growth when 4.0% to 5.0% (w/v) NaCl is present in the growth medium. Thus, the generation time of *S. aureus* when grown in media at 10°C, pH 6.5, and 0.5% (w/v) NaCl is 12 hours, while the addition of 4.5% (w/v) NaCl under the same growth conditions increases the generation time to 14.6 hours. In contrast, the generation time of

Table 5-9 Effect of Suboptimal Conditions of pH and Sodium Chloride on Generation Time of Foodborne Pathogens in Media

Organism	*Substrate*	*Temperature (°C)*	*NaCl (% w/v)*	*pH*	*Generation Time (hours)*	*Source of Data*
A. hydrophila	nutrient broth	5	0.5	4.7	27.2	86
		5	3.5	7.0	13.8	
		10	0.5	4.7	17.1	
		10	3.5	7.0	9.5	
B. cereus (psychrotrophic)	tryptone soya broth	5	0.0	6.0	32.4	Walker et al. (unpubl.)*
		5	2.0	7.0	27.7	
		10	0.0	5.0	12.5	
		10	5.0	7.0	7.9	
E. coli 0157:H7 4-strain cocktail	tryptone soya broth	10	0.5	5.5	8.4	Sutherland et al. (unpubl.)†
		10	2.5	6.7	12.5	
		10	2.5	5.7	14.4	
		15	0.5	4.5	7.4	
		15	4.5	6.5	8.1	
L. monocytogenes	tryptone soya broth	5	6.0	7.4	21.1	Sutherland et al. (unpubl.)†
		5	0.5	5.3	27.7	
		5	4.5	5.6	43.3	
		10	8.0	7.4	9.4	
		10	0.5	4.6	12.0	
		10	4.5	5.7	14.5	
Salmonellae	tryptone soya broth	10	0.5	5.1	15.4	Sutherland et al. (unpubl.)†
		10	4.5	6.6	15.5	
		10	3.8	6.0	24.1	
		15	0.5	5.0	4.7	
		15	4.5	6.6	7.5	
Staph. aureus	brain heart infusion	10	0.5	5.9	19.6	Sutherland et al. (unpubl.)†
		10	4.5	7.0	14.6	
		10	4.5	6.5	16.7	
		15	0.5	4.8	9.8	
		15	13.5	6.6	13.1	
		15	6.5	4.7	38.3	
Y. enterocolitica	brain heart infusion	5	0.5	4.8	35.1	Sutherland et al. (unpubl.)†
		5	4.5	6.97	17.0	
		5	3.5	5.8	25.7	
		10	0.5	4.53	17.97	
		10	6.5	6.96	28.0	
		10	4.5	5.44	28.0	

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†Unpublished data of Sutherland, J.P., et al., used in preparation of Food MicroModel. Reproduced by permission of the Ministry of Agriculture, Fisheries and Food, UK.

Y. enterocolitica is increased from 9 hours at 5°C, under optimum conditions of pH and NaCl, to 17 hours when 4.5% (w/v) NaCl is included in the medium.

Data presented in Table 5-10 demonstrate the effect on growth of foodborne pathogens of inclusion of carbon dioxide in the headspace of polystyrene trays containing filter pads saturated with medium containing foodborne pathogens, to simulate conditions in modified atmosphere packaged (MAP) foods. Calculation of the "inhibition index" (by dividing the generation time in the absence of CO_2 by that at a particular CO_2 concentration) shows that the growth rate of *E. coli* O157:H7 was little affected by concentrations of CO_2 up to and including 40% (w/v) at 10°C. At a concentration of 60% (w/v) CO_2 at 10°C, the generation time was reduced by one-third and 80% (w/v) CO_2 in the headspace prevented growth. However, at 15°C the generation time of *E. coli* O157:H7 was unaffected by even 80% (w/v) CO_2 in the headspace. *Y. enterocolitica* appears somewhat more inhibited by CO_2 in the environment than *E. coli* O157:H7. Gram-positive organisms such as *S. aureus* and *B. cereus* (mesophilic strains) appear to be more sensitive than Gram-negative fermentative organisms to CO_2, and this sensitivity is enhanced at temperatures near the minimum for growth.

This relationship between temperature and CO_2 concentration is significant in the ecology of MAP

Table 5-10 Effect of Carbon Dioxide on Generation Time of Selected Pathogenic Bacteria Growing in Nutrient Media at Low Temperatures

Organism	*Growth Medium*	*Temperature (°C)*	*NaCl (% w/v)*	*pH*	*CO_2 (% w/v)*	*GT* (hours)*	*Inhibition Index†*
B. cereus (mesophilic)	tryptone soya broth	10	0.5	7.0	0	12.6	—
					10	16.3	0.77
					40	19.1	0.66
		15	4.5	7.0	0	5.9	—
					10	6.0	1.0
					40	8.7	0.68
E. coli O157:H7 (4-strain combination)	tryptone soya broth	10	0.5	7.0	0	8.9	—
					10	8.9	1.0
					20	9.1	0.98
					40	9.4	0.95
					60	13.0	0.68
		15	2.5	7.0	0	8.1	—
					60	8.5	0.95
					80	8.6	0.94
Staph. aureus	brain heart infusion	10	0.5	7.0	0	16.2	—
					30	21.8	0.74
					50	40.1	0.40
					80	ng	0.0
		15	2.5	7.0	0	3.5	—
					30	5.7	0.61
					80	21.4	0.16
Y. enterocolitica	brain heart infusion	5	0.5	7.0	0	9.2	—
					30	13.9	0.66
					50	19.9	0.46
					80	22.8	0.40
		15	2.5	6.4	0	2.9	—
					30	4.1	0.71
					50	4.4	0.66
					80	7.3	0.40

*GT: generation time.

†Inhibition index: GT at 0% CO_2/GT at specific CO_2 concentration.

Source: Unpublished data of Sutherland, J.P., et al. Used in preparation of Food MicroModel. Published by permission of the Ministry of Agriculture, Fisheries and Food, UK.

foods, because the Gram-negative spoilage microflora is inhibited by CO_2, Gram-negative microaerophilic pathogens may be able to grow relatively unchecked, eg, for fresh meat the normal MAP contains 20% to 30% (w/v) CO_2, with the balance of oxygen for color maintenance. This concentration of CO_2 fails to inhibit growth of *E. coli* O157:H7 at 10°C, and also possibly salmonellae, while growth of *Y. enterocolitica* would be only slightly inhibited. It would therefore seem a reasonable hypothesis that modified gas atmospheres may effectively select for Gram-negative microaerophiles, several of which are foodborne pathogens.[59] This emphasizes the requirement for assiduous temperature control of MAP foods by manufacturers, distributors, and retailers of food and also by consumers.

Data presented in Table 5-11 show the effect of another common preservative, sodium nitrite ($NaNO_2$), on the generation times of salmonellae and *L. monocytogenes*. Systematically increasing $NaNO_2$ concentrations reduced the growth rate of *L. monocytogenes* at 10° and 20°C when conditions of pH and NaCl were slightly suboptimal (pH 6.0, NaCl concentration 4.0% w/v). Comparison of the inhibition indices shows that even 50 mg L^{-1} $NaNO_2$ has a noticeable effect on the growth rate, particularly at 10°C. At 20°C, under the same conditions of NaCl and pH value, the degree of inhibition caused by systematically increasing concentrations of $NaNO_2$ was less evident. Thus, good temperature control is essential for foods that contain $NaNO_2$ as a a preservative, including cured meats and particularly mild-cured products such as frankfurters and some types of bacon and ham.

Under optimal conditions of NaCl and pH value, the growth rate of salmonellae at 10°C was not noticeably affected by 50 mg L^{-1} of $NaNO_2$ (inhibition index equal to 1.0; Table 5-11), but 100 mg L^{-1} of $NaNO_2$ was noticeably inhibitory. However, little additional reduction in growth rate was achieved by further increasing the concentration of $NaNO_2$.

5.8 CONCLUDING REMARKS

The requirement for good and consistent refrigeration of perishable foods throughout the various stages of the chill chain, from manufacture, distribution, and retail to consumer use is demonstrated by the information contained in this chapter. The data suggest that even relatively small increases in temperature can cause a significant increase in growth rate, particularly among spoilage organisms and so-called low temperature pathogens, eg, *L. monocytogenes*, *Y. enterocolitica*, psychrotolerant *B. cereus*, and nonproteolytic *Cl. botulinum*. Furthermore, foodborne pathogens not normally regarded as able to grow at refrigeration temperatures, including salmonellae and *E. coli* O157:H7, are able to multiply, albeit slowly, at a temperature of 8°C. The present-day requirement for long shelf lives for chilled foods could

Table 5-11 Effect of Sodium Nitrite on Generation Time of Pathogenic Microorganisms Growing in Media at Low Temperatures

Organism	*Substrate*	*Temperature (°C)*	*NaCl (% w/v)*	*pH*	*NO_2 (mg L^{-1})*	*GT* (hours)*	*Inhibition Index†*
L. monocytogenes	tryptone soya broth	10	4.0	6.0	0	8.8	—
					50	19.6	0.45
					100	30.1	0.29
		20	4.0	6.0	0	2.0	—
					50	2.9	0.69
					100	4.3	0.47
					200	5.4	0.37
Salmonellae	tryptone soya broth	10	0.5	7.0	0	9.1	—
					50	8.9	1.0
					100	14.8	0.61
					150	16.4	0.55
					200	17.5	0.52

*GT: generation time.

†Inhibition index: GT at 0 mg L^{-1} NO_2/GT at NO_2 concentration.

Source: Courtesy of J.P. Sutherland et al., Ministry of Agriculture, Fisheries and Food, UK.

mean that foodborne pathogens may multiply significantly during long storage of a product at temperatures greater than about 6°C.[11,47,126,153]

Given the above concerns, it is therefore perhaps surprising that current refrigerated storage legislation is not more stringent. Regulations in the US state that refrigerated foods should be maintained at 7.2°C or below as appropriate for the food involved.[24] In the UK, the Food Safety (Temperature Control) Regulations introduced in England and Wales in 1995 stipulate that the maximum temperature permitted for storage of chilled foods is 8°C.[128] This is also the maximum temperature permitted in Sweden, while in Denmark, chilled meat and poultry products must be stored at temperatures of 5°C or less, with a maximum of 2°C for "special" meat and poultry products, eg, those that are modified atmosphere packaged. Some countries have resisted introducing legislation on temperatures on the grounds that it is not possible in practice to maintain them, but instead issue guidelines of an advisory nature.[17] With regard to the data presented in this chapter and our increased understanding of microbial growth at low temperatures, it would appear apposite to reevaluate current refrigeration practices and establish internationally agreed upon standards for the chill preservation of perishable foods.

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6 Freezing

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6.1 INTRODUCTION

The commercial production of frozen food probably started with ices and ice cream. A Florentine chef was said to have produced ices of different flavors for each day of the celebration of the wedding of Catherine de Medici to the French dauphin in 1534, and it is reported that by 1576 there were 250 master ice makers in Paris.[111] An anonymous manuscript on *L'art de Faire des Glaces* (The Art of Preparing Ice Cream) was written in about 1700, and a treatise on *The Art of Making Frozen Desserts* appeared in Paris in 1768.[67] In the United States (US) in 1865, there are records of the freezing of fish by placing them in pans surrounded by ice and salt.[37] In about 1880, refrigeration machines began to be used to freeze fish, and by 1900 fish freezing had become an important industry. The first reported successful shipment of frozen beef in the US was in 1867. In 1878, the first cargo of frozen meat was transported successfully from Australia to England. Freezing of poultry and fish began at about the same time. Freezing of shellfish and eggs developed later, that of small fruit and berries began in about 1905. The birth of the modern frozen food industry is regarded as dating from 1928, with developments in freezing technology by Clarence Birdseye[83] and in 1929 Birdseye frozen foods were placed on the retail market. Experimental work on freezing of vegetables was recorded in 1917; in 1929 it was recognized that in order to produce frozen vegetables of good quality it was necessary to heat the vegetables briefly before freezing in order to inactivate enzymes that could cause deterioration during frozen storage. This resulted in successful processes, and freezing of vegetables became commercially important in 1937. After 1945, satisfactory methods were developed for the concentration of citrus juices, which resulted in commercial production of frozen citrus juices. In addition to fresh foods, the production of frozen cooked and prepared foods has become a major part of the frozen food industry.

By the mid-1980s, frozen food accounted for approximately 40% of the food consumed in the US

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whereas in Europe the percentage ranged from 10% to 25%.[95] In 1996, retail sales of frozen foods in the United Kingdom (UK) were valued at £4.6 billion, and the worldwide frozen food market was valued at £51.6 billion.[42]

In the UK in 1996 the percentage by value of retail sales of different frozen foods was estimated as follows: meat and poultry, 24%; fish, 14%; ice cream, 12%; meat products, 11%; frozen potatoes, 8%; vegetables, 8%; desserts, 7%; meat ready meals, 7%; pizzas, 6%; vegetarian, 3%.[42]

Foods are usually subjected commercially to temperatures of –10°C or colder for one of the following reasons: (1) to preserve the quality and wholesomeness, (2) to create a special structure and consistency, eg, ice cream, frozen yogurt, or (3) to carry out certain production processes. Quick-frozen foods are defined as foods "that have undergone a freezing process known as quick-freezing whereby the zone of maximum crystallization is crossed as rapidly as possible depending on the type of product"[102] and that are maintained at –18°C or colder with a minimum of fluctuations. Other frozen foods such as butter stored in bulk and large cuts of meat or poultry are maintained at –12°C or lower. Ice cream is usually stored at lower temperatures than other frozen foods. According to European legislation, after freezing and temperature stabilization, quick-frozen foods should be kept at temperatures of –18°C or lower except during transport and local distribution, when they may be exposed for brief periods to temperatures not higher than –15°C.[22,23,24,85,102,103] During long-term storage, frozen foods should ideally be kept in primary cold stores in which the air temperature is between –20° and –28°C, and secondary cold stores should be designed to give similar air temperatures.[56,85] It is recommended that frozen fish be stored at –30°C or below in order to maintain high quality.[97] During retail display, it is good practice to keep quick-frozen foods at a temperature of –18°C, as far as possible, but not warmer than –12°C.[22,102] These regulations and recommendations do not apply to products that are not labeled "quick frozen" nor to ice cream or any other edible ice. It is recommended that main factory cold stores for ice cream be well below –18°C,[53] eg, below –30°C,[67] and that the temperature for delivery should not exceed –18°C.

Refrigerators and home freezers in several countries use the International Standards Organization (ISO) system of star marking to designate the temperature of the frozen food compartment. One star denotes a test temperature of –6°C, at which most frozen foods can be stored for up to 1 week.[56] Two stars denote a test temperature of –12°C at which most frozen foods can be stored for up to 1 month, and three stars denote a test temperature of –18°C at which most frozen foods can be stored for up to 3 months. There are few data on the practical storage life of frozen foods but approximate information is shown in Table 6-1.

In general, frozen foods have a good record of safety and quality. The minimum temperature for growth of most bacteria that cause spoilage of meat and other foods is, for practical purposes, probably in the range of –2° to –3°C and that for molds is probably about –8°C; growth at these temperatures is very slow (Section 6.5). Many microorganisms, however, can survive well in frozen foods, and several large outbreaks of disease, as well as sporadic cases, have resulted from the consumption of ice cream or of previously frozen food. After thawing, surviving microorganisms can multiply rapidly in these foods, depending on the temperature, and this can result in safety and spoilage problems. It is clear that strict control of raw materials and control of hygiene by Good Manufacturing Practice (GMP) are required during production in order to ensure the safety as well as the quality of these foods.

6.2 THE PRODUCTION OF FROZEN FOOD

The treatment of food before freezing determines the quality after freezing; the treatments that are needed to obtain the required quality are described in detail elsewhere.[56,64,90,97] In addition to affecting the sensory quality of foods, these treatments influence the microbiology of the frozen food. Before freezing,

Table 6-1 Approximate Storage Life of Groups of Foods at –18°C and –24°C

Product	*Practical Storage Life (months) at –18°C*	*Practical Storage Life (months) at –24°C*
Fruits	18–24	>24
Vegetables	6–24	15–>24
Meats and poultry	10–24	15–>24
Seafood	5–9	9–>12
Butter and cream	12–>24	14–>24
Ice cream	6	24
Cakes and raw dough	12–15	18–24
Breads	3	—

Source: Adapted with permission from *Recommendations for the Processing and Handling of Frozen Foods*, 3rd ed., © 1986, International Institute of Refrigeration, Paris.

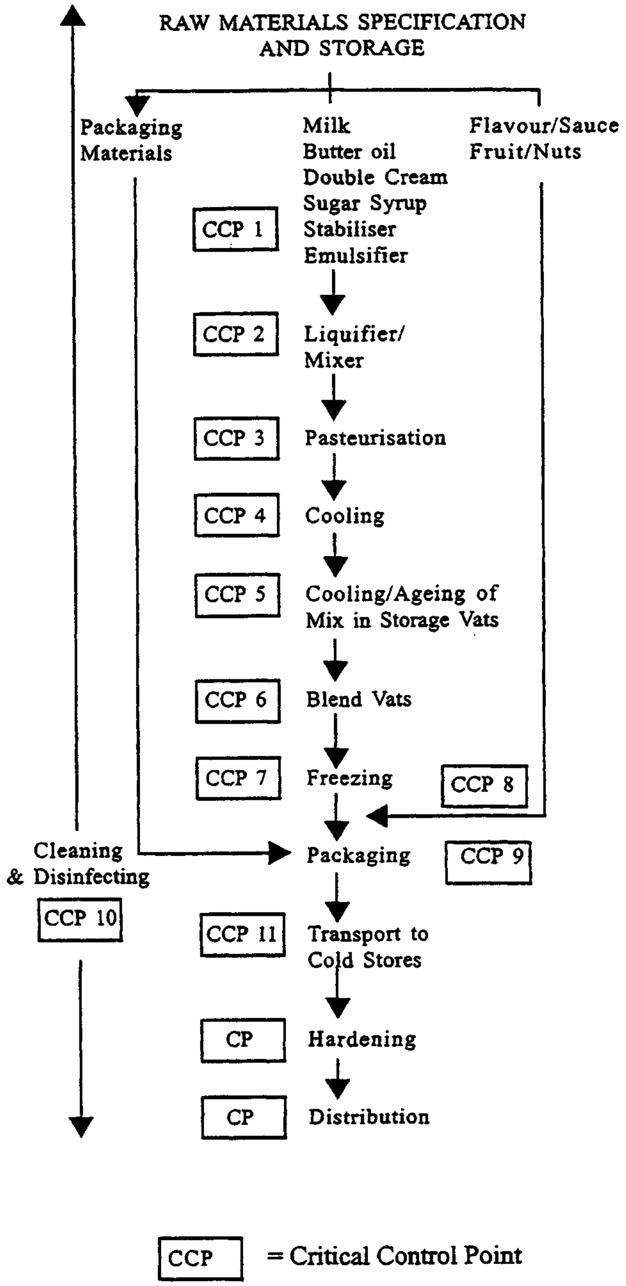

Figure 6-1 Outline of the Production of a Flavored, Bulk, Hard Ice Cream Containing Sauce with Fruit and Nuts. *Source:* Reprinted with permission from *Code of Practice for the Hygienic Manufacture of Ice Cream,* © 1995, Ice Cream Alliance, Nottingham, UK.

beef and lamb carcasses should be stored long enough for rigor mortis to occur, when the pH will have fallen to 6.0 or lower. If prerigor muscle is cooled to below 10°C, the muscle contracts because the sarcoplasmic reticulum can no longer bind calcium ions efficiently, a phenomenon called *cold shortening.* This can be prevented by allowing a period of time during which the metabolism of muscle glycogen to lactic acid results in a fall in pH to below pH 6, or by electrical stimulation of the meat. Before freezing it is usually desirable to bleed fish.[97] The majority of vegetables are blanched before freezing.[6] This is mainly in order to inactivate peroxidase, catalase, and lipoxygenase enzymes, which could cause deterioration of flavor and texture during long-term frozen storage. Blanching is done by passage of the vegetables through steam or hot water, usually between 75°C and 95°C for 1 to 10 minutes depending on the size of the vegetable pieces. Vegetables such as bell peppers, cucumbers, leeks, herbs, Jerusalem artichokes, onions, swedes, and turnips (and possibly carrots) will retain high quality without blanching provided that they are maintained at temperatures not warmer than –18°C.

Freezing of ice cream is not merely a means of preservation but is part of the design of the product. Production is described in detail elsewhere[67,91] and a Code of Practice for Hygienic production has been published in the UK.[53] The process for production of hard ice cream is outlined in Figure 6-1. Ice cream is made from a liquid mix that contains milk, cream, water, milk-solids-not-fat, milk fat or other fat, sugars, emulsifying and stabilizing agents, flavor, and color. Following a 1947 outbreak of typhoid fever in Wales, caused by contaminated ice cream, regulations were introduced in the UK requiring heat treatment of ice cream mix. The present UK regulations require that ice cream mixture must not be kept for longer than one hour at a temperature that exceeds 7.2°C before being pasteurized or sterilized. The conditions for pasteurization of ice cream mix in the UK are heat treatment (1) at not less than 65.6°C for at least 30 minutes, (2) at not less than 71.1°C for at least 10 minutes, or (3) at not less than 79.4°C for at least 15 seconds.[104] Slightly greater heat treatments are recommended in the US.[67] Sterilized ice cream must be prepared by heating the mixture to a temperature of not less than 148.9°C for at least 2 seconds. In each case the mix must then be cooled to a temperature of not more than 7.2°C within 1.5 hours of being heated (or in the US, cooled to <5°C) and must be kept at such temperature until the freezing process is begun. Regulations differ slightly in different countries.

After heat treatment, the ice cream mix is homogenized to reduce the size of fat globules, cooled, and stored for up to 24 hours at a temperature of about 3°C. It is then frozen by lowering the temperature rapidly to about –5° to –7°C with agitation and simultaneously air is incorporated. When the ice cream emerges from the freezer, it is either dispensed for sale as "soft-serve" ice cream or packaged in bulk or small packs, hardened at low temperature, eg, –20° to –40°C, and sold as hard ice cream.

Equipment and methods used for the freezing of foods are summarized in Table 6-2 and factors that influence the choice of methods are outlined by Persson and Londahl.[83]

During the freezing of a food any particular location in the food passes through three stages of temperature change: (1) prefreezing, (2) freezing, and (3) reduction to the storage temperature.[83] The *prefreezing stage* starts when the food is subjected to the freezing process and lasts until the beginning of ice formation. The *freezing stage* is that in which the temperature at the specific location remains almost constant because the heat extracted is causing the main part of the water to change to ice. The initial freezing point depends on the composition of the food and is below 0°C; in the case of a highly salted product, such as bacon, it can be below –7°C. The *stage of reduction to storage temperature* is the period during which the temperature is reduced from that maintained during freezing to the required storage temperature. The *freezing time* is defined as the time from the start of the prefreezing stage until the food has reached the final temperature.[56] The *freezing rate*, measured in °C h^{-1}, is the difference between the initial and final temperatures divided by the freezing time. It can be assessed by the speed of movement of the ice front (in cm h^{-1}) through a product; this speed is faster near the surface of the product than near the center. The freezing process can be regarded as complete when the equilibrium temperature has reached the intended storage temperature (–12°C or lower for frozen foods in general; –18°C for quick-frozen foods). The most rapid rate of freezing reached is probably equivalent to a rate of fall in temperature of about 100°–200°C min^{-1} for small particles, eg, peas, and at the surface of other foods, whereas in many cases the rate of fall in temperature will be much slower than this.

Table 6-2 Equipment and Methods Used for Commercial Freezing of Foods

Type of Freezer	*Comments*
Contact freezers	
Plate freezers	Product is pressed between hollow metal plates through which refrigerant is circulated. Used for whole, gutted fish; packaged fish fillets; blocks of fish; and packaged meat products.
Contact belt freezers	Product moves on a belt with refrigerant circulated on the other side of the belt. Can freeze and form product into pellets. Used for chopped spinach purees, fruit pulps, egg yolk, sauces, and soups.
Drum and rotary freezers	Used for pastes, eg, chopped spinach paste; thick liquids; and flat, thin items such as fish.
Air blast freezers	
Blast room	Cold storage room with extra refrigeration capacity. Used for bulk products, eg, butter, beef quarters, and fish.
Tunnel freezer	Loading may be manual or mechanized. Used for most types of food, particularly meat carcasses and 20–30 kg cartons of meat.
Belt freezers	Belts may be multitier or spiral. Used for meat patties, fish fillets, chicken portions, vegetable products, and packaged products such as ready meals.
Fluidized bed freezers	Particles are frozen and conveyed by an upward stream of cold air. Used for sliced or diced vegetables, fruits, meat products, and cooked shrimps.
Cryogenic freezers	
Liquid nitrogen freezers (–196°C at 1 bar)	Liquid nitrogen evaporates to gas at –196°C. The liquid nitrogen is sprayed into the freezer and moves counter-current to the product. Used for meat cuts, fish fillets, shrimps, fruit berries, pies, and pastries.
Liquid carbon dioxide freezers (–78.5°C)	Solid CO_2 sublimes to vapor at –78.5°C. Liquid CO_2 under pressure is sprayed into the freezer and forms a snow, which then sublimes. Used for the same products as liquid nitrogen.
Combination methods	The product is surface frozen in liquid N_2, then passed to spiral belt freezer or fluidized bed freezer for complete freezing.
Form freezing	Liquids or semi-liquids are frozen into pellets. Liquid N_2 is sprayed into product to prefreeze. The frozen product is subjected to high pressure to form pellets, then freezing is completed.

Source: Data from D.F. Boast, The Technology of Freezing, in *Microbiology of Frozen Foods*, R.K. Robinson, ed., pp. 1–39, © 1985, Elsevier Applied Science; *Recommendations for the Processing and Handling of Frozen Foods*, 3rd ed., © 1986, International Institute of Refrigeration; P.O. Persson and G. Londahl, Freezing Technology, in *Frozen Food Technology*, C.P. Mallett, ed., pp. 20–58, © 1993, Blackie Academic and Professional.

6.3 PHYSICAL CHANGES DURING FREEZING

In addition to the formation of ice, the physical changes in foods during freezing include changes in concentration of solutes and in water activity, which affect the survival of microorganisms. Some of the principles underlying these changes can best be understood in relation to simple aqueous suspensions of microorganisms, but more complex changes may occur in the heterogeneous environment in foods. At 0°C the vapor pressure of pure water and of ice are the same. Low concentrations of solutes reduce the vapor pressure of an aqueous solution as is expressed by Raoult's law:

$$(p_0-p)/p_0 = n_1/(n_1 + n_2)$$

where p_0 is the vapor pressure of pure solvent, p is the vapor pressure of the solution, n_1 is the number of moles of the solute, and n_2 is the number of moles of the solvent. The number of gram molecular weights of water in one kilogram, n_2, = 55.49. The above equation indicates the depression of water activity $(1-a_w)$.

For a one molal aqueous solution

$$n_1/(n_1 + n_2) = 1/56.49 = 0.0177,$$

therefore, for a one molal solution $(p_0-p)/p_0 = 0.0177$, ie, the vapor pressure of a one molal ideal solution will be 1.77% lower than that of the pure solvent, and the solution will be in water vapor pressure equilibrium with a relative humidity of 98.23%; this would be expressed as a water activity (a_w) of 0.9823.[69]

In a one molal ideal solution, the freezing point is lowered by 1.86 K (=1.86°C), ie, the molal depression constant K_f for water, is 1.86 K (= 1.86°C).[10,74] This applies only to simple ideal solutions at low concentrations. For concentrated ionic solutes, the relationship between molality and vapor pressure and between molality and freezing point is more complicated (Chapter 7).

In the case of most foods, freezing begins between −1° and −3°C.[10,54] During cooling below this temperature either supercooling occurs, in the absence of nucleation, or ice crystals begin to form. The formation of ice crystals results in concentration of solute in the remaining solution and a consequent reduction in freezing point of that solution. At each temperature, a certain amount of ice is in equilibrium with a certain concentration of unfrozen solution. The vapor pressure of ice is, therefore, equal to the vapor pressure of the unfrozen solution. The a_w of this unfrozen solution and of the food itself is, therefore, given by the ratio of the vapor pressure of ice to that of water at the temperature concerned.[94] At −5°, −10°, and −15°C, the corresponding values of a_w are 0.9526, 0.9074, and 0.8642, respectively; the a_w in frozen foods can only be controlled by changing the temperature. The relationship between the a_w and temperature for mixtures of ice and aqueous solutions is shown in Figure 6-2.

Eventually all the water that can be crystallized as ice freezes, leaving the solute(s) and its water of hydration, ie, the solution is saturated with respect to the solute. In suitable conditions, further reduction of temperature results in the solidification of the remaining solution. The lowest temperature at which the solution remains liquid is called the eutectic temperature.

If the eutectic temperature is fairly high, eg, −4°C in the case of lactose, conditions will be relatively favorable for formation of a eutectic mixture. If the eutectic temperature is well below the initial freez-

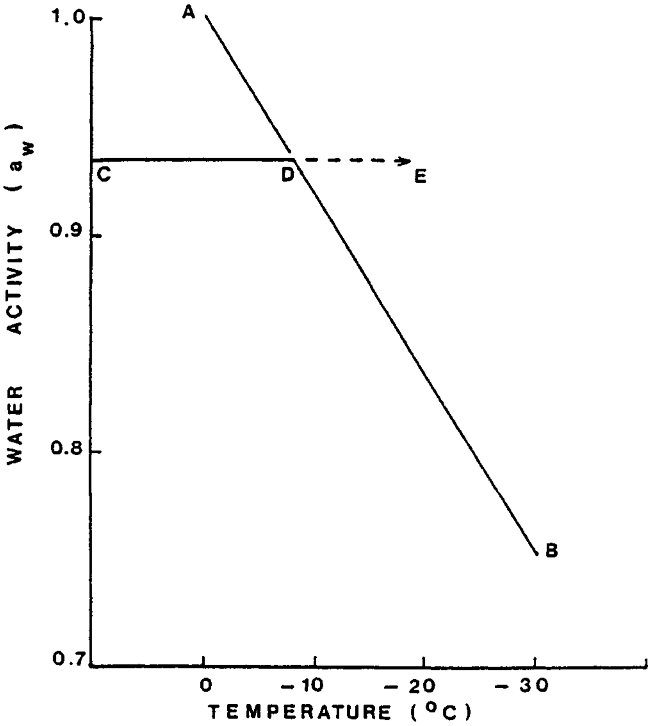

Figure 6-2 The Relationship between Temperature and Water Activity in Partially Frozen Ice/Water Mixtures.[26] The line AB refers to ice and water mixtures. The effect of reduction of temperature on the a_w of a high-solute liquid is shown by line CDB where the freezing point is depressed to D, or alternatively if supercooling occurs the solution remains liquid to temperature E. *Source:* Copyright © 1985, R. Davies.

ing point of the food sample, as in the case of the majority of foods, the unfrozen solution may be very viscous at that temperature, and this viscosity may so reduce the movement of molecules as to prevent the formation of a eutectic mixture. In this case, the unfrozen solution remains in a metastable, supersaturated state, which is probably the situation in most frozen foods.[41] During further cooling this supersaturated, unfrozen phase may either (1) persist as a supersaturated, viscous liquid or (2) be transformed into a glass. This is illustrated for a simple system in Figure 6-3.

In frozen foods that contain a supersaturated, viscous liquid (case 1), physical and chemical changes that are commercially significant can occur during storage, whereas frozen foods where a glassy state occurs (case 2), the rates of physical and chemical change are much lower, thus reducing deterioration during storage. Glass transition temperatures, Tg^1 values, for some food components and foods are shown in Tables 6-3 and 6-4.

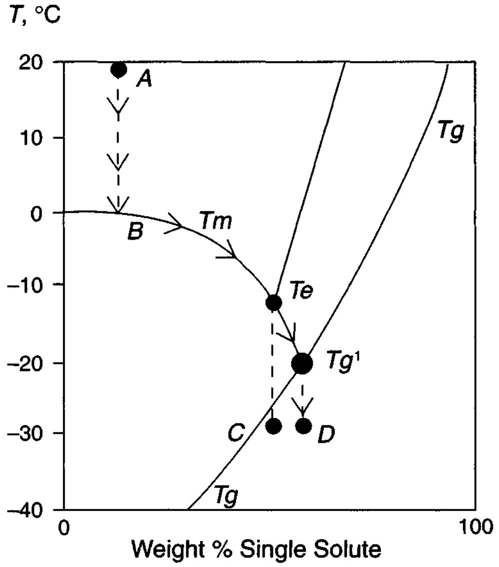

Figure 6-3 Simplified Temperature–Composition State Diagram for an Aqueous Binary System. *Tm* = melting (freezing) temperature; *Te* = eutectic temperature; *Tg* = glass transition temperature; Tg^1 = subzero *Tg* of the maximally freeze-concentrated amorphous solute/unfrozen matrix surrounding the ice crystals in a frozen solution. If a eutectic forms the composition will be unchanged by further cooling (*Te–C*). Further cooling from Tg^1 will not change the composition (Tg^1–*D*). *Source:* Reprinted with permission from O. Fennema, Frozen Foods: Challenge for the Future, *Food Australia,* Vol. 45, pp. 374–381, © 1993, Australia Institute of Food Science & Technology.

Table 6-3 Glass Transition Temperatures (Tg^1) and Molecular Weights of Food Components

Solute	*Molecular Weight (daltons)*	*Glass Transition Temperature* Tg^1 *(°C)*
Glycerol	92.1	−65
Xylitol	152.1	−46.5
Glucose	180.2	−43
Sucrose	342.3	−32
Maltotriose	504.5	−23.5
Maltoheptaose	1153.0	−13.5
Gelatin	30,000–300,000	−9.5 to −12.5
Waxy maize	$>10^6$	−4

Source: Reprinted with permission from O. Fennema, Frozen Foods: Challenge for the Future, *Food Australia*, Vol. 45, pp. 374–381, © 1993, Australia Institute of Food Science & Technology.

The Tg^1 values for vegetables are within the range of storage temperatures for frozen foods, while the Tg^1 values for fruits are rather lower. *Tg* values for meat are likely to be in the range −9.5° to −12.5°C.[41] Rates of physical and chemical change in these frozen foods are likely to be much lower below than above these temperatures.

Other changes that can occur during freezing of foods include change in pH, ranging from 0.3–2.0 pH units, and loss of gasses such as oxygen and carbon dioxide. The formation of ice is associated with an increase in volume and can result in a build-up of substantial pressure, which can cause cracking of some foods.

During freezing, cells and tissues may be protected from damage by certain solutes, a process known as *cryoprotection.* In addition to affecting the quality of frozen foods, cryoprotectants are also likely to protect microorganisms against damage during freezing. Some foods naturally contain cryoprotectants and these are present in foods such as dairy desserts. Cryoprotectants that may be present naturally in, or may be added to, frozen foods include sugars, salts, polyhydric alcohols, and glycerol, the last being the most effective. The commercial production of frozen surimi (a minced, white fish product that has been subjected to washing processes to remove soluble materials) depends on the addition of cryoprotectants to preserve the gel-forming characteristics.[75] The cryoprotectants used in surimi are typically a mixture of sucrose, 4%(w/w); sorbitol, 4%(w/w); and tripolyphosphates, 0.2%(w/w).

Table 6-4 Glass Transition Temperatures (Tg^1) for Various Foods

Food	Tg^1 *(°C)*
Fruits	
Apple	
Red delicious	–42
Granny Smith	–41
Banana	–35
Blueberry	–41
Peach	–36.5
Strawberry, 10 cultivars	–33 to –41
Tomato	–41.5
Fruit Juices	
Apple	–40.5
Orange, various samples	–37.5
Pineapple	–37.5
Vegetables	
Broccoli	
stalk	–26.5
head	–11.5
Carrot	–25.5
Cauliflower	
stalk	–25
head	near 0°C
Corn, sweet, blanched	–9.5
Pea	–25
Potato, Russet Burbank	–12
Spinach	–17
Meat	not found
Milk, whole	–22
Ice cream, vanilla	–31 to –35

Source: Reprinted with permission from O. Fennema, Frozen Foods: Challenge for the Future, *Food Australia*, Vol. 45, pp. 374–381, © 1993, Australia Institute of Food Science & Technology.

6.4 THE EFFECT OF FREEZING ON MICROORGANISMS

6.4.1 General Comments

Experiments using simple aqueous suspensions have enabled investigation of the factors that affect survival of microorganisms during freezing and thawing, and their enumeration. In many cases, these experiments have been made with the aim of preservation of microorganisms by freezing, but they provide information that is relevant to the survival of microbes in frozen foods. At temperatures above 0°C, a sudden decrease in temperature can cause "cold shock," associated with damage to some bacteria. This usually only affects microorganisms in the logarithmic phase of growth and may only be of minor importance in freezing foods. While experiments in aqueous suspensions can provide information relevant to microorganisms within the matrix of foods, microbes at the surface of solid foods may also be subjected to drying effects during freezing.

6.4.2 Mechanism of the Effect of Freezing

During freezing of aqueous suspensions of microorganisms the microbes become concentrated in the unfrozen portion of the solution where they are subjected to the effect of concentrated solutes. As the temperature is reduced and more water is frozen, the increase in solute concentration in the unfrozen liquid results in diffusion of water from the microbial cells.

During freezing and thawing, factors that might be expected to cause damage to microorganisms include (1) low temperature, (2) extracellular ice formation, (3) concentration of extracellular solutes, (4) intracellular ice formation, and (5) concentration of intracellular solutes. The influence of these factors will be affected by the rate of freezing and thawing.

Temperatures below 0°C are not necessarily lethal to microbes provided that the cells and their environment do not freeze.[68] For example, about 100% of cells of *Saccharomyces cerevisiae* survived in suspensions cooled to –16°C when the extracellular water was supercooled and rapid cooling of a suspension of *Escherichia coli* to –9°C or –10°C caused no loss of viability provided that the cells remained supercooled.[68] In nonhalophilic microbes the molal concentration of intracellular solutes is usually less than 1 and the freezing point would, therefore, be between 0° and –2°C. In practice, during freezing of a suspension of microbes the external water freezes before the cell contents, which usually remain unfrozen above –5°C and often above –10°C. This may be due in part to supercooling of intracellular water[68] and in part to an increase in concentration of intracellular solutes. As the temperature is reduced, more water is frozen and the increase in solute concentration in the unfrozen liquid tends to result in diffusion of water from the microbial cells. Thus, the supercooled cell is subjected to two competing effects: (1) the increased concentration of extracellular solutes, which tends to cause intracellular water to diffuse from cells, and (2) the lowered temperature, which increases the tendency to formation of intracellular ice. If water cannot diffuse out of the cell sufficiently rapidly it will become increasingly supercooled and likely to freeze. Thus, the response of microorganisms to freezing is determined largely by the permeability of the cell to water, its size, and its surface-area-to-volume ratio. Injury to *S. cerevisiae* during freezing is probably due both to an increased intracellular concentration of solutes and to intracellular freezing.[68] Very low rates of cooling will lead to intracellular concentration of solutes but not to intracellular ice formation, whereas at cooling rates of between 10°C and 100°C, there is evidence of formation of intracellular ice crystals. In the case of *E. coli,* intracellular freezing is only likely to occur at freezing rates of 100°C min^{-1} or higher, and most low temperature damage during freezing at lower rates than this, and during frozen storage, is thought to be due to the effect of concentrated extracellular solutes, especially sodium chloride.[68] In some species of molds, eg, *Penicillium expansum,* freezing in growth medium at a cooling rate of 2°C min^{-1} resulted in shrinkage of hyphae, whereas at a higher cooling rate, eg, of about 20°C min^{-1}, resulted in intracellular ice formation.[73] For a range of species the rate of cooling during freezing that resulted in the presence of intracellular ice differed between 4°C min^{-1} and 53°C min^{-1}. Some species, including *Aschersonia alleyrodis*, *Phytophthora nicotianae,* and *Volvariella volvacae,* showed shrinkage at all the cooling rates examined (10°–100°C min^{-1}), and intracellular ice was not observed. Cryogenic freezing may give cooling rates of 100°C min^{-1} at the surface of some foods, but usually the cooling rate during freezing is likely to be substantially lower than this.

Injury and death of cells during freezing and frozen storage depends on time and temperature. In general, at very low temperature injury accumulates slowly, the rate of injury increasing with increasing temperature to reach a maximum near, but not at, the freezing point. The rate of injury during thawing may not be the same as that during freezing.

6.4.3 Factors That Affect the Survival of Microorganisms during Freezing and Thawing

Studies of the survival of microorganisms in simple aqueous systems have shown the ways in which specific factors can influence survival. Factors that may be involved are shown in Exhibit 6-1.

Exhibit 6-1 Factors Affecting the Survival of Microorganisms during Freezing and Thawing

- Type and strain of microorganism
- Nutritional status
- Phase of growth
- Composition of cooling and freezing medium
- Rate of cooling
- Holding temperature
- Time held at low temperature
- Rate of warming to melting point
- Dilution medium prior to viable count
- Method of determination of viable count
- Medium used for determination of viable count

Type and Strain of Microorganism

Bacterial spores are usually very resistant to freezing and thawing, therefore, a high proportion survive in most conditions. In general, Gram-positive bacteria are more resistant than Gram-negative organisms. Following rapid freezing of spores of *Bacillus mesentericus* to −70°C in water and subsequent thawing, about 90% of the spores remained viable.[47] The survival rates of vegetative bacteria in the same conditions were as follows: *Pseudomonas aeruginosa*, 18%; *Escherichia coli* 58%; *Staphylococcus aureus*, 96%, while that of *Saccharomyces cerevisiae* was 11%.

It is difficult to assess quantitatively the effect of freezing on the survival of filamentous fungi because of the complexity of the "colony-forming unit." In favorable conditions high rates of survival can occur. After cooling suspensions of a range of fungi in growth medium to −196°C at optimum cooling rates (from 1°C min^{-1} to 200°C min^{-1} depending on species), the estimated percentage recovery ranged from 27% to approximately 100%.[73] Free-living amoebae and most ciliated protozoa are relatively susceptible to freezing.

Nutritional Status and Phase of Growth

The nutritional status and phase of growth of microorganisms can influence their resistance to freezing and may have a small effect on their survival during freezing of foods. Studies of chemostat-grown cells of *E. coli* indicated a general correlation between carbohydrate content and survival during freezing.[15] In the case of frozen starter cultures and frozen doughs, a high rate of survival of microbes is desirable, and growth conditions may be controlled to ensure good survival. Growth of a commercial strain of baker's yeast, *Saccharomyces cerevisiae,* with controlled substrate feeding (fed-batch culture) was the major factor that conferred resistance to freezing; strong aeration in addition to fed-batch culture gave the highest resistance with up to 92% survival of cells freeze thawed to −50°C for 10 minutes in water.[44] In cultures grown in highly aerobic conditions, a high trehalose content was correlated with resistance to freezing. Trehalose has been recognized as a particularly effective cryoprotective carbohydrate; its effect has been attributed to membrane stabilization. The fact that the correlation between trehalose content and cryoresistance only occurred in cells grown in optimum oxygenation indicated the existence of another cryoprotective factor more important than trehalose.

Composition of Cooling and Freezing Medium

The effects of increasing concentrations of extracellular solutes on bacteria during freezing are minimized if the cells are suspended in distilled water. The survival of some Gram-negative bacteria is lower in NaCl solution than in water and death rates during storage are higher at temperatures above the eutectic point of the medium than below it. When a suspension of *E. coli* in NaCl, 0.85% (eutectic temperature −21.8°C) was cooled to between −7° and −18°C, the survival after 24 hours was reported as 10%, whereas at lower temperatures 30% to 40% of cells survived.[68] Washed, chemostat-grown (ie, log phase) cells of a strain of *E. coli*, frozen to temperatures below −20°C in saline, showed much lower viability than those frozen in water (Figure 6-4).[14]

Cells frozen to −70°C at a range of cooling rates showed much lower viability in saline than in water (Figure 6-5).[14]

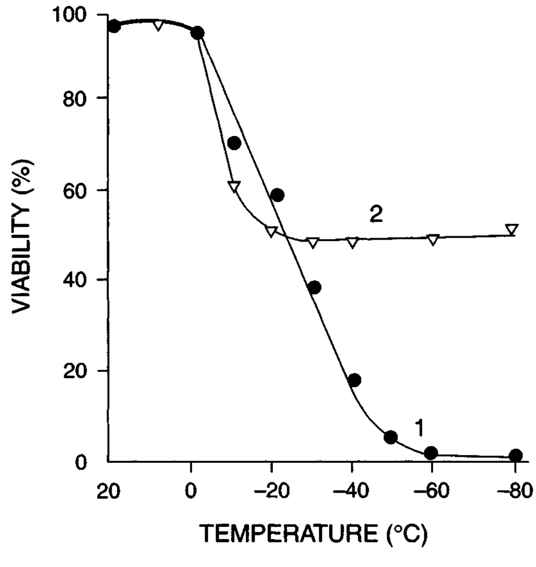

Figure 6-4 Effect of Temperature of Freezing in Distilled Water (curve 2) and in 0.85% Saline (curve 1) on Viability of *E. coli*. The bacterial suspensions were cooled at 25°C min^{-1} to various temperatures and thawed rapidly. Viability was determined by slide culture. *Source:* Reprinted with permission from P.H. Calcott and R.A. Macleod, Survival of *Escherichia coli* from Freeze-Thaw Damage: A Theoretical and Practical Study, *Canadian Journal of Microbiology*, Vol. 20, pp. 671–681, © 1974, NRC Research Press.

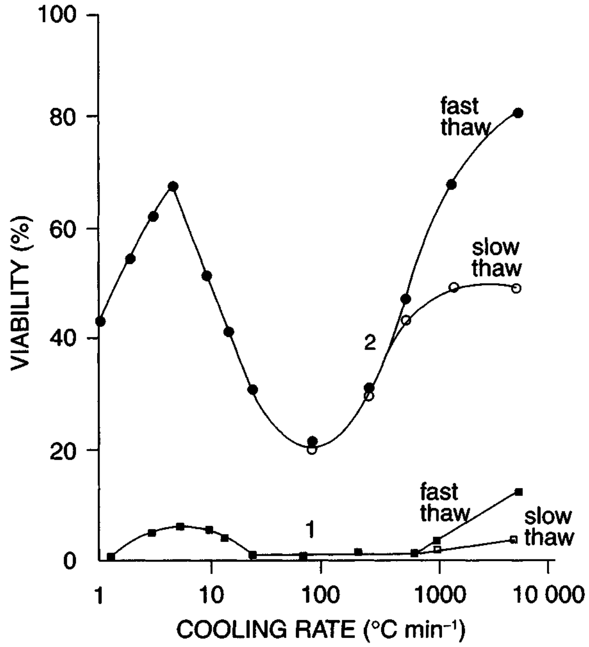

Figure 6-5 The Effect of Cooling and Warming Rates on Survival of *E. coli* Suspended in Distilled Water (curve 2) or in 0.85% Saline (curve 1). Suspensions were frozen to at least −70°C. Viability was determined by slide culture. *Source:* Reprinted with permission from P.H. Calcott and R.A. Macleod, Survival of *Escherichia coli* from Freeze-Thaw Damage: A Theoretical and Practical Study, *Canadian Journal of Microbiology*, Vol. 20, pp. 671–681, © 1974, NRC Research Press.

Glycerol and sucrose can act as cryoprotectants and protect *E. coli* against the lethal effects of freezing in water (Figure 6-6) and in saline (Figure 6-7).[14]

This protective effect may be due both to the fact that the fraction of the molarity contributed by the NaCl will be reduced and to the fact that the nonelectrolytes may protect the cells against the effect of the electrolyte.[68]

In contrast to *E. coli, Streptococcus faecalis* was resistant to the presence of NaCl during freezing,[16] and saline had a protective effect on *Saccharomyces cerevisiae.*[68]

Survival of bacteria may be reduced in an acidic medium. Thus, adjustment of the pH of trypticase soy broth from pH 7.3 to pH 3.8 with HCl decreased survival of *Staphylococcus aureus* during freezing to −30°C by eight-fold and a high proportion of the survivors were sublethally injured.[71]

The Effect of Rate of Cooling

Figure 6-5 shows the effect of rate of cooling during freezing of *E. coli* in water to −70°. Maximum

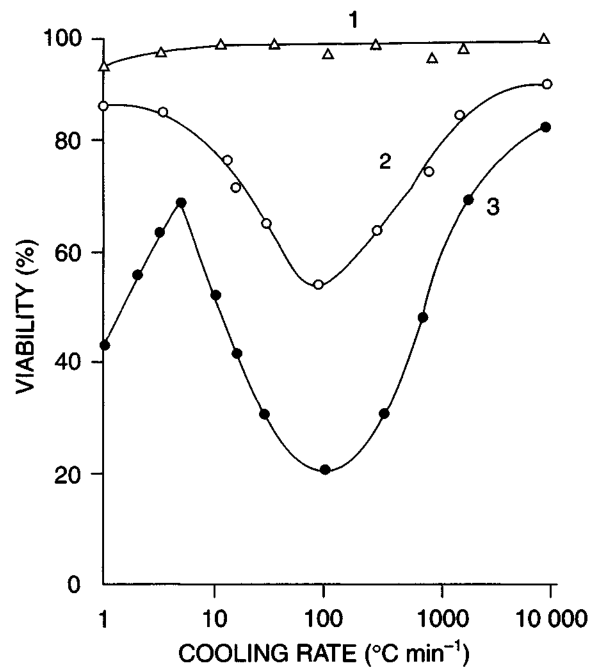

Figure 6-6 Protection of *E. coli* by Glycerol from Freeze-Thaw Damage in Distilled Water. Procedure as in Figure 6-5, except that the bacteria were frozen in distilled water + 3% glycerol (curve 1), + 1% glycerol (curve 2), or distilled water alone (curve 3). Viability was determined by slide culture. *Source:* Reprinted with permission from P.H. Calcott and R.A. Macleod, Survival of *Escherichia coli* from Freeze-Thaw Damage: A Theoretical and Practical Study, *Canadian Journal of Microbiology,* Vol. 20, pp. 671–681, © 1974, NRC Research Press.

survival occurred at a cooling rate of 6°C min^{-1}, and the viability dropped at cooling rates lower and higher than this. As discussed earlier, the rate of freezing of foods will usually be lower than 100°C min^{-1} and often lower than 10°C min^{-1}. The effects of different rates of cooling may be obscured by the effects of protective factors.

Holding Temperature and Time Held at Low Temperature

The majority of reports indicate that the rate of death of bacteria during frozen storage is usually greatest just below the freezing point. The sensitivity of bacteria to freezing was found to be correlated with sensitivity to storage in the frozen state.[47] When spores of *Bacillus mesentericus* in water were stored for 133 days at temperatures from –1° to –20°C, between 50% and 100% remained viable, whereas the decrease in viability of the other bacteria tested was *Staphylococcus aureus* < *Escherichia coli* < *Pseudomonas aeruginosa,* the decrease in viability being greatest at the highest temperature (Figure 6-8).

Usually the decrease in viability is very small at temperatures of –60°C and below.

Rate of Warming to Melting Point

After relatively low rates of cooling, such as are used in freezing of foods, the rate of warming is reported to have little effect on survival of bacteria or yeasts.[13] After rapid cooling, fast warming is more protective than slow warming.

Techniques Used for Determination of Viable Count

Freezing and thawing can result in sublethal damage to microorganisms, making them unable to multiply on selective media (Chapter 15). In many cases, therefore, it is necessary to resuscitate damaged microorganisms to estimate the number that remain viable in frozen foods (Section 6.6). Usually suble-

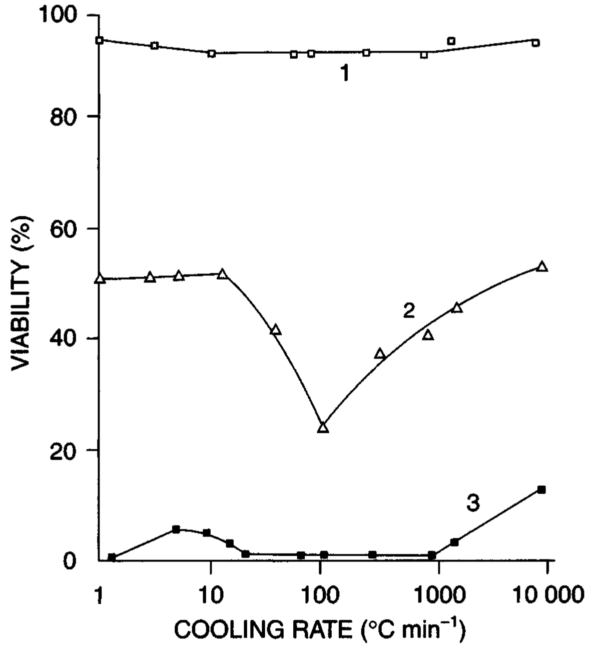

Figure 6-7 Protection of *E. coli* by Glycerol from Freeze-Thaw Damage in 0.85% Saline. Procedure as in Figure 6-5, except that the bacteria were frozen in saline + 3% glycerol (curve 1), + 1% glycerol (curve 2), or saline alone (curve 3). *Source:* Reprinted with permission from P.H. Calcott and R.A. Macleod, Survival of *Escherichia coli* from Freeze-Thaw Damage: A Theoretical and Practical Study, *Canadian Journal of Microbiology,* Vol. 20, pp. 671–681, © 1974, NRC Research Press.

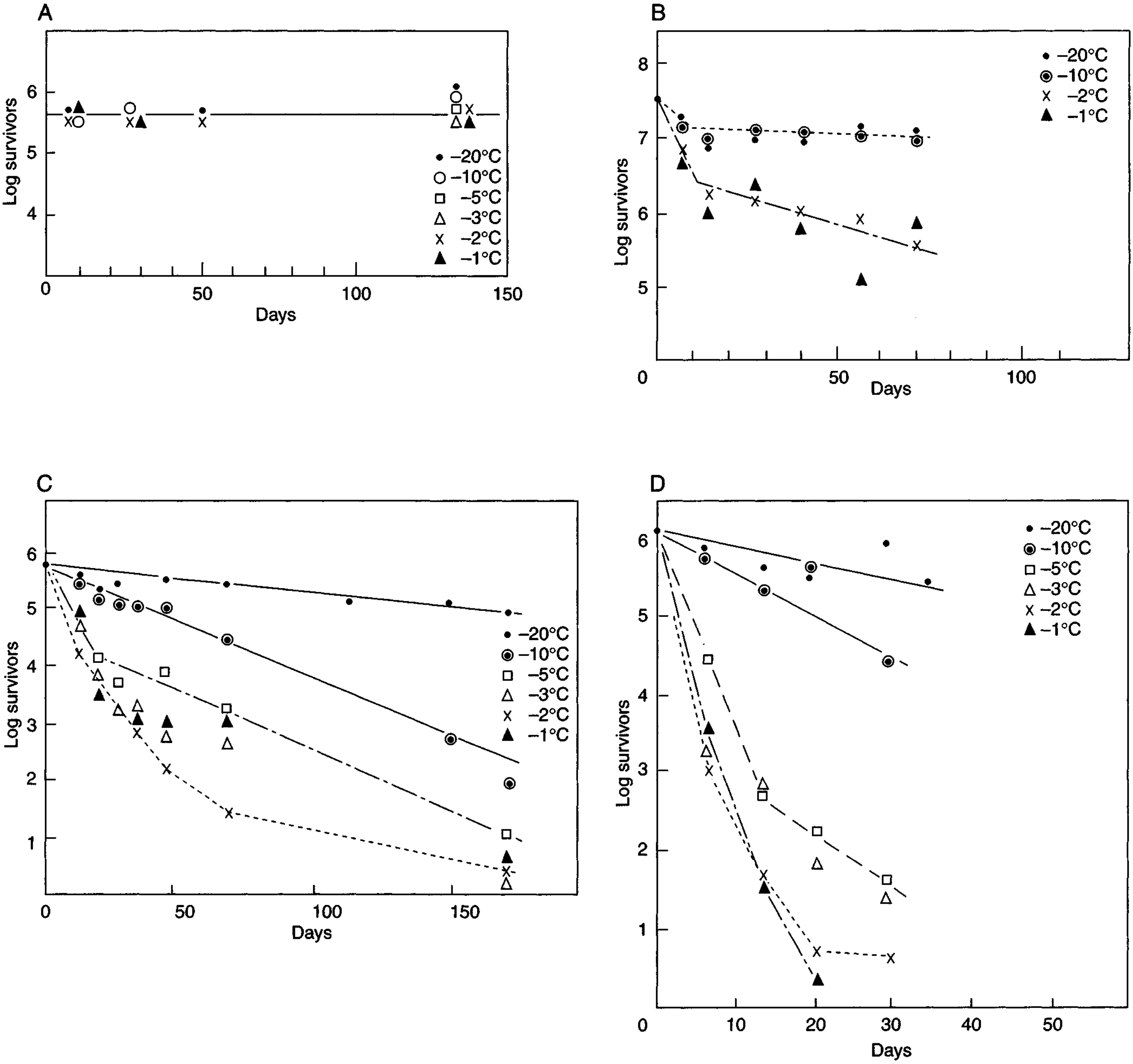

Figure 6-8 The Effect on Bacteria of Storage in Frozen, Aqueous Suspension. (**A**) spores of *Bacillus mesentericus*, (**B**) *Staphylococcus aureus*, (**C**) *Escherichia coli*, and (**D**) *Pseudomonas aeruginosa*. *Source:* Reprinted with permission from R.B. Haines, The Effect of Freezing on Bacteria, *Proceedings of the Royal Society*, Vol. 124B, pp. 451–463, © 1938, Royal Society.

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thally damaged bacteria grow more readily on complex nutrient media than on minimal media.

6.5 EXAMPLES OF THE EFFECT OF FREEZING ON MICROORGANISMS ASSOCIATED WITH FOODS

The following examples are chosen to illustrate the survival of different types of microorganisms, including the most resistant and the least resistant foodborne pathogenic bacteria, in foods.

6.5.1 Bacterial Spores

The resistance of spores to freezing and frozen storage was illustrated by work showing that quick or slow freezing of spores of *Clostridium botulinum* type B in phosphate buffer or in pea juice (pH 7.1) had

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no effect on viability; repeated freezing and thawing also had little or no effect on the toxin.[57] Both *C. botulinum* spores and toxin survived freezing in fruits and vegetables and storage for 1 year at –16°C.[115] Whereas in food, *C. botulinum* toxin appears to retain activity after freezing and thawing, crystalline type A toxin in 0.05 M acetate buffer pH 4.2, which retained its activity at +4°C for 1–2 years, was completely detoxified by freezing.[93]

6.5.2 Gram-Positive, Vegetative Bacteria

In a study involving both Gram-positive and Gram-negative bacteria, it was reported in 1938 that after inoculation of a high number of the organisms into ice cream, *Brucella abortus* and *Salmonella enteritidis* survived during storage at –23.2°C for 7 years.[114] Other strains of *Brucella* and *Salmonella* and *Mycobacterium tuberculosis hominis* and *M. tuberculosis bovis* were still viable after between 4 and over 6 years. The author pointed out that because of the high inoculum used and the fact that commercial ice cream (in the US) was made from pasteurized milk, the practical importance of these results should not be emphasized. It is interesting, however, to recognize the possible longevity of these organisms in ice cream.

Reports that vegetative cells of *Clostridium perfringens* were found in fresh meat more frequently than the spores gave rise to studies of the survival of vegetative cells of this organism in frozen meat products.[112] In fresh meat products inoculated with vegetative cells and spores of *C. perfringens* and frozen at –29°C, the number of viable spores remained practically unchanged for 42 days. The number of vegetative cells decreased by between 38% and 75% in 1 day, then declined slowly to give a decrease of about 89.5% in 42 days. In Ellner's medium frozen at –29°C, the number of viable spores remained unchanged, whereas the number of vegetative cells decreased by 99.5% in 42 days. A further study gave similar results, showing that after freezing in a meat medium at –23°C and storage for 14 days, the numbers of viable vegetative cells decreased by 94%.[43]

After freezing in whole milk and storage at –20°C for up to 231 days the number of viable *Staphylococcus aureus* decreased by only about 10%.[7] During storage of raw, minced beef at –22°C for 4 months the numbers of *S. aureus* decreased by 91%, ie, by about one log cycle,[71] and in frozen beef or chicken stored at –18°C for 168 days, the numbers also decreased by about one log cycle.[117] In lean ground beef with a pH in the range 4.6–6.3 the numbers of viable *S. aureus* were reported to remain stable throughout four cycles of freezing in a mixture of dry ice/ethanol and thawing, but in beef adjusted to pH of 4.2, a decrease of just over one log cycle occurred.[29] *Staphylococcus* enterotoxin is not inactivated by freezing.

Freezing and frozen storage of *Listeria monocytogenes* for 1 month at –18°C in phosphate buffer or in Tryptose broth caused death of 54% to 87% of cells

Table 6-5 The Effect of Temperature of Freezing, Frozen Storage, and Suspending Medium on Survival of *Listeria monocytogenes* Strain Scott A

			Suspending Medium	
Treatment	*Freezing*	*Storage*	Phosphate Buffer, *0.1 M, pH 7*	Tryptose Broth
1	–18°C, 30 minutes	–18°C, 4 weeks	87% cells dead 10.3% cells injured* 2.7% cells uninjured	54% cells dead 21% cells injured 25% cells uninjured
2	–198°C, 1 minute	–198°C, 4 weeks	0 cells dead 0 cells injured 100% cells uninjured	15.5% cells dead <0.8% cells injured 83.7% cells uninjured
3	–198°C, 1 minute	–18°C, 4 weeks	60% cells dead 14% cells injured 26% cells uninjured	61% cells dead 17.2% cells injured 21.8% cells uninjured

*% of cells that were injured, determined from the number of bacteria that formed colonies on Tryptose agar and the number that formed colonies on Tryptose agar plus 16% NaCl.

Source: Data from S.E. El-Kest et al., Fate of *Listeria Monocytogenes* During Freezing and Frozen Storage, *Journal of Food Science*, Vol. 56, pp. 1068–1071, © 1991, Institute of Food Technologists.

and injury of others, whereas freezing and storage for 1 month at −198°C had little lethal effect (Table 6-5).[35,36] Further death and injury occurred during longer storage at −18°C but not at −198°C.

The addition of up to 4% of glycerol, milk fat, or lactose and up to 2% casein to phosphate buffer had a protective effect during freezing and storage at −18°C.[34] A protective effect of food was shown by work in which the *L. monocytogenes* was added to ground beef (pH 5.84) that had been irradiated to reduce the background microflora[80]; after freezing to −18°C and subsequent storage at this temperature for 14 weeks the number of *L. monocytogenes* was unchanged. Comparison of numbers on Tryptose phosphate agar + 1% sodium pyruvate (designed to allow multiplication of damaged bacteria), and on Tryptose phosphate agar + 5% NaCl (designed to allow multiplication of undamaged bacteria only) showed no evidence that cells were damaged. Similar results were obtained in other foods with a pH higher than 5.8 but in tomato soup (pH 4.74) the numbers of *L. monocytogenes* tended to decline slightly during storage at −18°C and a high proportion of the cells were sublethally damaged; this resulted in lower counts on selective than on nonselective media.

L. monocytogenes has been found in many samples of ice cream, ice milk, and related frozen products,[35] and in the US this has led to many voluntary, Class 1 recalls of products. Following inoculation into ice cream to give about 0.5 colony-forming units per ml of mix, *L. monocytogenes* strain Scott A showed no significant decrease in numbers during storage at −18°C for 5 months, and could be recovered readily by selective as well as nonselective enrichment procedures.[62] The survival (measured by counting on a selective medium) of *L. monocytogenes* during freezing of ice cream and storage at −18°C was reduced significantly by the incorporation of sufficient nisin to give a concentration of 400–500 IU g^{-1},[27] but the possible presence of sublethally damaged listerias was apparently not investigated.

Because of the short, seasonal nature of the milking period for ewes and goats, freezing is used as a means of storage of cheese curd after drainage, and of ewes' and goats' milk in Greece, France, Italy, and Spain in order to extend the period of manufacture of cheese. Experiments in which *L. monocytogenes* was added to ewes' milk showed that a high proportion of the bacteria (>95% of strain Scott A and and 40%–50% of strain CA) could survive in the milk during frozen storage at −18° or −38°C for over 7 months.[81] In Feta cheese curd samples (pH 5.43) numbers of strain Scott A decreased by *ca.* 50% in the center of the cheese and by *ca.* 22% on the surface after storage at −18°C for 7.5 months, whereas numbers of strain CA at the center and at the surface had decreased by *ca.* 98%.

These results indicate that Gram-positive bacteria often show a survival rate of between 10% and over 90% during freezing and frozen storage in nonacidic foods, whereas in foods with a low pH or that contain inhibitors a lower rate of survival may occur.

6.5.3 Gram-Negative Bacteria

Although, in general, Gram-negative bacteria are less resistant to freezing than Gram-positive organisms, nevertheless they may survive well in frozen foods depending on the protective effect of those foods. The survival of pathogens such as *Salmonella* species and verocytotoxigenic *Escherichia coli* in frozen foods is of particular concern because of the low numbers that can cause disease.

In fish frozen at −22°C and stored at −17.8°C for over a year, 10% of the inoculum of *S. typhimurium* remained viable.[84] Comminuted beef protected *S. typhimurium* during storage at −20°C and no marked decrease in viable count was detected after 90 days, although daily freeze-thaw cycling from −20°C to −4°C to −20°C resulted in a decrease of about 1,000-fold in viable count.[45] Heat denatured meat suspensions were less protective, and adjustment of the pH of such a suspension to 5.8 or 4.25 increased inactivation in 20 days at −20°C by about 100-fold compared with very little inactivation at pH 7.5. After storage in beef sausage and in cooked, minced beef at between −18° and −20°C for up to 10 weeks, the viable count of *S. typhimurium* on a nonselective medium had decreased by less than 10-fold.[8] Comparisons of viable counts on a nonselective medium and on a selective medium showed that before freezing 5% of the bacteria in minced beef and 36% in beef sausage were sublethally damaged, whereas after freezing and frozen storage the proportion had increased to 30% in minced beef and 45% in the beef sausage mix.

Following inoculation of *E. coli* O157:H7 into ground beef, which was then formed into patties, maintained at −80°C for 30 minutes and stored at −20°C for up to 9 months, little if any decrease in viable numbers occurred during freezing and storage.[31]

Campylobacter jejuni is usually relatively sensitive to adverse conditions but in a survey of fresh and frozen meat and poultry samples collected at the point of slaughter, *C. jejuni* was isolated from 12.1% of fresh samples and from 2.3% of frozen samples.[105] The organism has been detected in frozen chickens from supermarkets and can survive for extended periods in frozen meats.[1] After inoculation into ground beef, which was then frozen at –15°C, the number of *C. jejuni* had decreased approximately 1,000-fold at the first time of sampling (3 days) but then remained unchanged for up to 14 days.[106] Survival was increased by the addition of cryoprotective agents such as 10% glycerol. In brucella broth frozen at –18°C, the numbers of viable *C. jejuni* decreased by about 100-fold within 5 days; the presence of 3% NaCl in the broth reduced the number of survivors by about 50-fold.[1] In raw or cooked chicken and in beef and cod at –18°C, the decrease in viable count was less than that in the broth and NaCl had less effect.

Studies of the survival of *Vibrio* species in frozen seafood have indicated a marked decrease in numbers during storage. In a study of the survival of *Vibrio cholerae* in frozen seafood homogenates log-phase cells were inoculated into the sterilized homogenate and viability after storage was assessed by an MPN method after enrichment in alkaline peptone medium.[87] The use of log-phase bacteria might be expected to give a minimum estimate of resistance to freezing. After freezing and storage at –20°C for 21 days the log number of viable *V. cholerae* had decreased by approximately 3.0, 5.5, and 5.0 in oyster, shrimp, and crab meat, respectively.

After inoculation into oysters and freezing at –15°C or –30°C, the numbers of *V. parahaemolyticus* decreased by up to 10-fold during freezing and then in an approximately log linear way during storage.[58] After storage for 100 days at –15°C the log numbers of viable vibrios had decreased by approximately 3 to 4, while at –30°C the log numbers had decreased only by about 1.5. From studies of the survival of *V. parahaemolyticus* in an oyster meat broth at –18°or –24°C, times for reduction in numbers from 10^2–10^4 g^{-1} to below about 1 g^{-1} were calculated as about 9 to 10 weeks.[77]

In shrimp homogenate inoculated with *V. parahaemolyticus* or with *V. vulnificus* then frozen and stored at –20°C, the numbers of these bacteria had decreased by about 10^6 in 40 days.[11] On the basis that the number of these bacteria in market fish and crustacea is usually fewer than 10^3 g^{-1}, and the fact that the infectious dose of *V. parahaemolyticus* is considered to be relatively high, the authors suggested that frozen storage of shellfish at –20°C offered considerable protection to the consumer.

Following inoculation of *V. vulnificus* into freshly harvested, shucked oysters, which were then packed in plastic bags under air or under vacuum and frozen at –20°C in a laboratory freezer, the number of viable *V. vulnificus* decreased by about 4 logs in 30 days under air and by over 3 logs under vacuum, whereas after 70 days a similar decrease, of just under 5 logs, occurred in both types of packaging.[82] Substances that protected *V. cholerae* during storage at –20°C were obtained from prawn shells and were much more active than other cryoprotective substances such as 10% glycerol.[96]

Despite the fact that some of the above reports indicated a relatively rapid decline in the numbers of *Vibrio* species during frozen storage of seafood, in a survey of 114 samples of frozen seafoods available in Taiwan, *V. cholerae* was recovered by enrichment from 14.9%, *V. parahaemolyticus* from 15.8%, and *V. vulnificus* from 3.5%.[120] In tryptic soy broth plus 1% NaCl frozen rapidly and stored at –30°C, the numbers of viable *V. cholerae* decreased by 100-1,000-fold in the first day and declined more slowly for a further 2 days, whereas freezing in shrimp homogenate resulted in a gradual decrease of approximately 10-fold in 3 days.

6.5.4 Yeasts and Molds

Although some experiments have shown that *Saccharomyces cerevisiae* was less resistant to freezing than *Escherichia coli*, this resistance may be increased by control of conditions during growth. The resistance of this yeast to freezing is of particular importance in the production of frozen, yeast-leavened doughs. After growth in fed-batch cultures in strongly aerobic conditions the organism showed *ca.* 100% survival in dough frozen to –50°C at a freezing rate of 20°–42°C min^{-1}.[44]

6.5.5 Viruses

Evidence from experiments in which foods were inoculated with viruses indicates that these can be very resistant to freezing. In various foods frozen at –20°C poliovirus and type B1 and B6 coxackieviruses decreased by a factor of less than 10 during storage for 5 months.[65] In oysters frozen at –36°C and stored at –17.5°C, approximately 91% of poliovirus was viable after 2 weeks and 10% survived after 12 weeks.[30] In prawns frozen and stored at –17.5°C, more than 10% of polio 1 virus survived for 300 days.[39]

6.5.6 Protozoa

There is little published information concerning the ability of protozoa to survive in frozen foods. An anecdotal report described infection with *Cryptosporidium* species apparently as a result of the ingestion of frozen tripe contaminated with oocysts.[89]

Oocysts, 10^5 in 100 μl water, were killed by snap freezing in liquid nitrogen but when exposed to slow freezing at −22°C, 33% remained viable (as judged by *in vitro* excystation and exclusion or inclusion of two fluorogenic vital dyes) after 21 hours and 1.8% remained viable after 775 hours.[89] After freezing 8×10^6 oocysts in 400 μl deionized water, all oocysts frozen at −70°C appeared to be noninfectious and cryoprotectants failed to prevent this inactivation.[40] When oocysts were frozen at −10° for up to 168 hours, −15° for up to 24 hours, and −20°C for up to 5 hours a proportion remained infective. The authors concluded that ice cubes prepared in the freezing compartment of a home refrigerator cannot be relied on to be free of *Cryptosporidium* even if frozen for longer than 24 hours, and that if oocysts are frozen in a medium containing organic materials or minerals or exposed to temperatures higher than −10°C they might survive for a longer period.

Freezing can be used to inactivate cysts of *Toxoplasma gondii,* which may be present in meat, particularly pork. These cysts are more readily inactivated than are the larvae of *Trichinella spiralis.*[60] Following inoculation into pigs, which were killed 42 to 69 days after inoculation, and the meat stored at −6.7°C for up to 11 days, viable cysts were still present in the meat as determined by inoculation of mice, but after storage at a temperature of −9.4°C or lower for 64 minutes, meat was no longer infective.[60]

6.5.7 Parasites

Because meat is liable to be eaten undercooked, freezing has been used for many years to inactivate the nematode *Trichinella spiralis* in pork and pork products. From studies of the effect of freezing on meat from pigs infected with *T. spiralis,* it was concluded that freezing to −29°C using cryogenic equipment would immediately render pork and pork products free of this parasite.[92] For this purpose the US Code of Federal Regulations[21] specifies freezing at −15.0° for 20 days, −23.3° for 10 days, or −28.9°C for 6 days for pork less than 15.2 cm diameter and longer for products with a greater diameter. Further information is available concerning the combinations of time and temperature required.[59] The European Union also requires that every imported pig carcass that has not been inspected for *Trichinella* must be submitted to a freezing procedure, eg, storage at −25°C for 240 to 480 hours, depending on the size of the cut or carcass, in order to destroy the larvae.

In sausages and certain other pork products, the USDA Certification Program recommends freezing the meat for specified times in order to inactivate *Trichinella spiralis.*[21] In order to obtain precise time/temperature data a study was made in which pigs were inoculated with larvae of *T. spiralis,* slaughtered about 45 days after inoculation, the meat was homogenized, pressed to 2 mm thickness, packed into pouches, and frozen.[59] The concentration of larvae in the meat was estimated as >1,000 infective larvae per g. The survival of viable larvae was assessed by infectivity in rats. In these conditions, where heat transfer was very effective, maintenance at −23.3°C for 1 second with a come-down time of 5 minutes and a come-up time of 2 minutes resulted in complete destruction of infectivity.

Freezing is also used to kill the larvae of nematodes of the family *Anisakidae,* which can cause disease in humans as a result of the consumption of infected raw or inadequately cooked fish or other seafood. After freezing infected fish at −20°C, 100% inactivation of larvae was reported in 1 to 5 days.[28] In the Netherlands, where cases of infection have resulted from the consumption of "green herring," a fresh raw herring sometimes slightly salted, legislation requiring all freshly caught herring to be frozen prior to selling has been introduced as a means of control.[55]

6.6 THE MICROBIAL FLORA OF FROZEN FOODS

In order to detect viable microorganisms in frozen food by direct plating or enrichment there is a need to allow the recovery of sublethally damaged microbes. This is particularly important in the case of pathogenic bacteria that, if present, are likely to be found only in low numbers and therefore selective media are required to detect them. In tests for the presence or absence of pathogenic bacteria, particularly Gram-negative organisms, resuscitation is advisable before selective enrichment. Methods for resuscitation of damaged microorganisms are discussed in Chapters 15 and 62. In order to assess numbers of viable bacteria, samples can be plated on a membrane overlaid on a resuscitation medium and after incubation to allow recovery of damaged organisms the membrane is transferred to the surface of a selective medium.[2,52] In a cheaper, alternative method a diluted sample of the frozen food was transferred to a Petri dish to which a solid resuscitation medium (eg, a medium containing yeast extract, tryptone, sodium pyruvate, sodium glycerophosphate and magnesium sulphate) was added.[49] After a short incubation period, the medium was overlaid with a selective medium. Use of this type of method increased the numbers of *E. coli* detected in frozen meat and poultry products by a factor of at least 2 compared with counts directly in or on a selective medium.[49] The resuscitation medium of Hall[49] also gave a higher count of mesophilic, aerobic bacteria in frozen foods than Plate Count Agar, which is widely used in estimation of the number of these bacteria in frozen foods.[79]

Foods that are heat treated before freezing include vegetables, prepared meals, some meat products, and some fish products. In these foods many of the microorganisms present at freezing are derived from processing equipment, air, or personnel. In the case of foods that are not heat treated before freezing, the microorganisms on the frozen product will include a high proportion of those on the fresh food together with contaminants from the factory environment.

The blanching treatment given to many vegetables before freezing kills the majority of vegetative bacteria present and can reduce the count of aerobic bacteria from about 1×10^6 g^{-1} to about 10^2 g^{-1}.[70] The major groups of bacteria present on these vegetables after freezing differ from those that are present on fresh vegetables. In a large US study of peas, snap beans, and whole-kernel corn during preparation for freezing, the main groups of microorganisms in the final product were Gram-positive, catalase-negative cocci, which gradually became predominant in products after 15 or more days of processing.[99] These bacteria probably became established on equipment such as slicers, conveyers, inspection belts, and filling machines.[12] The cocci appeared to be heterofermentative leuconostocs, lactic streptococci, and enterococci; this last group was not considered to have any sanitary significance and similar bacteria have been isolated from fresh vegetables. In a study of frozen peas in the UK in the early 1960s, in which the number of aerobic bacteria was usually between 10^4 and 10^6 g^{-1}, most of the bacteria were of two species, *Leuconostoc mesenteroides* and *Streptococcus lactis*.[116]

In a further extensive study of frozen vegetables in the US in 1977 the majority contained between 10^4 and 10^6 aerobic bacteria g^{-1}.[98] The number of fecal coliform bacteria detected was between 5 and 200 g^{-1}.[100] *E. coli* was only recovered from 29% of the fecal coliform-positive samples, the majority of isolates being *Klebsiella pneumoniae* or other coliforms often found on raw vegetables.

On commercial samples of frozen chicken, prawns, peas, and sliced green beans the numbers of aerobic, mesophilic bacteria, assessed on Plate Count Agar without resuscitation, were between 2.25×10^4 and 2.25×10^5 g^{-1}.[79]

High numbers of microorganisms may be present in ice cream and similar products. In the UK, the following microbiological criteria are applied to ice cream (where n = number of sample units; c = number of sample units where the bacterial count may be between m and M; m = the threshold value, the result is satisfactory if the number of bacteria in all the sample units does not exceed m; M = the maximum number of bacteria, the result is unsatisfactory if the number of bacteria in one or more of the sample units exceeds M): an aerobic colony count, m = 100,000 g^{-1}, M = 500,000 g^{-1}, $n = 5$, $c = 2$; coliforms and *Staphylococcus aureus*, each $m = 10$ g^{-1}, $M = 100$ g^{-1}, $n = 5$, $c = 2$; *Salmonella*, absence in 25 g, $n = 5$, $c = 0$; *Listeria monocytogenes*, absence in 1 g, $n = 5$, $c = 0$.[53,104] A survey in the UK in 1993 of retail samples of ice cream and other edible ices showed that of about 1,000 samples of branded, hard ice creams only 1% had aerobic plate counts higher than 10^4 g^{-1} compared with 25% of 964 samples of unbranded hard ice creams (Table 6-6).[78] A low proportion of samples of unbranded hard ice cream and of soft ice cream did not meet the industry standards for *S. aureus*, *Salmonella* species, and *L. monocytogenes* and contained $>10^2$ *E. coli* g^{-1} or $>10^2$ *Bacillus cereus* g^{-1}, and one sample of "real-fruit" hard ice cream contained *S. enteritidis* PT4 (Table 6-7).

Packaged ice produced under conditions of poor hygiene may contain relatively high numbers of microorganisms. A survey in Iowa in 1991 showed that all of seven samples produced on the premises of a convenience store contained $>10^3$ bacteria ml^{-1}, and five of these contained $>10^4$ ml^{-1}.[76]

During the production of frozen foods, routine tests are used to monitor the general microbiological quality of the food and contamination with pathogenic bacteria, and to verify the effectiveness of hazard analysis critical control point (HACCP) control of the process. Methods of sampling and analysis were discussed in detail by Hall[48] and White and Hall.[118]

6.7 OUTBREAKS OF DISEASE ASSOCIATED WITH FROZEN FOODS

The incidence of illness due to the consumption of frozen foods is relatively rare, nevertheless these foods, in particular ice cream, have been responsible for some serious outbreaks and cases of disease (Table 6-8).

In the UK before the introduction in 1947 of statutory regulations regarding the heat treatment of ice

Table 6-6 Survey of Retail Samples of Ice Cream and Similar Products in the UK in 1993. Number (and %) of Samples with Counts of Aerobic Bacteria per Gram (on Plate Count Agar) within the Stated Ranges

		Range of Counts per Gram after Incubation at 30°C for 48 Hours						
Product	*Total Samples*	10^2–$<10^3$	10^3–$<10^4$	10^4–$<10^5$	10^5–$<10^6$	10^6–$<10^7$	$>10^7$	*Not Examined*
Ice cream bars (branded)	87	72 (83%)	14 (16%)	1 (1%)	0	0	0	0
Other hard ice cream	1,000	447 (45%)	296 (29%)	152 (15%)	75 (7%)	19 (2%)	9 (1%)	9 (1%)
Soft ice cream	964	330 (34%)	218 (23%)	197 (20%)	105 (11%)	78 (8%)	36 (4%)	0

Source: Adapted with permission from G. Nichols and J. de Louvois, The Microbiological Quality of Ice-Cream and Other Edible Ices, *PHLS Microbiology Digest*, Vol. 12, No. 1, pp. 11–15, © 1995, Public Health Laboratory Service.

cream mix, ice cream was frequently a vehicle for transmission of pathogenic microorganisms. Many outbreaks were caused by *Staphylococcus aureus* enterotoxin. *Staphylococcus aureus* from nasal and hand carriers contaminated the mix and multiplied and formed toxin before freezing. In one outbreak associated with ice cream, more than 700 people were affected by *S. aureus* enterotoxin.[51] The last major outbreak in the UK due to contaminated ice cream occurred in 1946, when about 210 cases of typhoid fever including four deaths were reported in Aberystwyth.[38] The ice cream was contaminated by the manufacturer who was a urinary excreter of *Salmonella typhi*, but had been declared free of infection in 1938. Following this outbreak, in 1947 legislation requiring pasteurization of ice cream mix was introduced in the UK.[51] Outbreaks and cases of disease due to the consumption of ice cream continue to be reported from many countries. Frequently these cases have been caused by *Salmonella* species and associated with the use of raw egg and failure to pasteurize the ice cream mix. Despite the fact that commercial production of ice cream in the UK is controlled by legislation that specifies pasteurization of the ice cream mix, this does not apply to homemade products, recipes for which in books and magazines often contain fresh eggs and involve little or no heat treatment.

In 1967 in the US, a common source outbreak of infection with *S. typhimurium* affected people at 14 separate banquets.[5] Epidemiological evidence indicated that the outbreak was due to an imitation ice cream produced by a single company that specialized in kosher desserts, and the product had been made from unpasteurized egg yolk that had not been heated during production of the ice cream. *S. typhimurium* was isolated from leftover samples of the frozen dessert and, together with two other species of *Salmonella*, from four unopened cans of unpasteurized, frozen, sugared egg yolks sampled from the manufacturer. It was estimated that 1,790 people who attended these banquets became ill, and the total number affected by products from this manufacturer may have been much higher.

During the period 1966–1976, 22 outbreaks involving 292 individual cases of salmonellosis associated with the consumption of homemade ice cream

Table 6-7 Survey of Retail Samples of Ice Cream in the UK in 1993. Number of Samples with *E. coli* or Specific Pathogens (numbers g^{-1})

Product	*Total Samples*	*E. coli* >3	*E. coli* $>10^2$	*S. aureus* $>10^2$	*B. cereus* $>10^2$	*L. monocytogenes*	*Salmonella species*
Ice cream bars, branded	87	0	0	0	0	0	0
Other hard ice cream	1,000	33	12	7	1	2	1
Soft ice cream	964	33	11	5	2	2	0

Source: Adapted with permission from G. Nichols and J. de Louvois, The Microbiological Quality of Ice-Cream and Other Edible Ices, *PHLS Microbiology Digest*, Vol. 12, No. 1, pp. 11–15, © 1995, Public Health Laboratory Service.

Table 6-8 Examples of Outbreaks of Disease Associated with the Consumption of Frozen Food

Outbreak	*Pathogen*	*Number of Cases*	*Number of Deaths*	*Food*	*Reference*
1945, UK	*Staphylococcus aureus*	700	NR*	ice cream	51
1946, Wales	*Salmonella typhi*	105	4	ice cream	38
1967, US	*Salmonella typhimurium*	1,790	NR	nondairy ice cream	5
1971, Trinidad	*Salmonella typhi*	132	0	unpasteurized ice cream	107
1980, Germany	*Salmonella enteritidis*	*ca.* 200	NR	ice cream	32
1982, US	*Salmonella typhimurium*	8	1	homemade ice cream	108
1983, UK	Hepatitis A virus	24	0	frozen raspberries	86
1987, UK	*Bacillus cereus*	6	0	homemade ice cream	101
1987, US	Norwalk virus	191	0	ice	17
1988, UK	*Salmonella enteritidis*	18	0	homemade ice cream	25
1988, Thailand	*Entamoeba histolytica* and *Giardia lamblia*	17	0	ice cream or ice	61
1991, UK	*Salmonella enteritidis* PT4	2	0	homemade ice cream	72
1991, Peru	*Vibrio cholerae*	unknown		ice	88
1991, US	*Vibrio cholerae*	4	0	frozen coconut milk (imported)	109
1993, US	*Salmonella enteritidis* PT 13a	12	0	homemade ice cream	18
1994, US	*Salmonella enteritidis* PT 8	*ca.* 22,400	NR	ice cream	50
1996, June Germany	*Salmonella enteritidis*	30	NR	ice cream	110
1996, August Germany	*Salmonella enteritidis*	237	NR	ice cream	3
1997, Germany	*Salmonella enteritidis*	90	NR	ice cream	4
1997, US	Hepatitis A virus	>150	NR	frozen strawberries (imported)	19
1997, US	*Escherichia coli* O157:H7	15	NR	frozen beef patties	20

*NR: not reported.

were reported to the US Centers for Disease Control.[46] *Salmonella typhimurium* was associated with 12 of these outbreaks, *S. enteritidis* with five, and five were associated with other salmonellae.

In an outbreak of typhoid fever in Trinidad in 1971, the majority of cases occurred in children aged 5 to 14, and epidemiological evidence implicated a commercially prepared ice cream made without pasteurization; it was suspected that an infected carrier of *S. typhi* was the source of the contamination.[107]

The consumption of homemade ice cream caused the death of a previously healthy teenager and severe illness in other members of a family in the US in 1982.[108] The ice cream contained about 10^6 *S. typhimurium* g^{-1}, and this was linked to the use of eggs from hens that carried *S. typhimurium* in the intestine and oviduct.

An outbreak in 1988 in the UK affected 18 out of 75 persons who attended a private function and were infected with *Salmonella enteritidis*; homemade ice cream prepared from raw shell eggs was implicated.[25]

In September 1991, the Public Health Laboratory Service in the UK received three reports of family outbreaks of *S. enteritidis* PT4 infection associated with the consumption of homemade ice cream.[72] In family outbreaks the food vehicle has often been consumed before an investigation of illness takes place, but in the 1991 UK outbreak shown in Table 6-8 the ice cream had been prepared using raw shell eggs and contained approximately 10^5 *S. enteritidis* PT4 per gram.[72]

An outbreak of illness due to *S. enteritidis* in the US in 1993 that affected 12 people was associated with the consumption of homemade ice cream made with raw eggs; *S. enteritidis* of the same phage type was isolated from patients and from the ice cream.[18]

The outbreak of infection with *S. enteritidis* in the US in 1994 that was attributed to a nationally distributed brand of ice cream, was reported as the largest common-vehicle outbreak of salmonellosis ever recognized in the US.[50] The pasteurized ice cream premix had been transported in tanker trailers that had previously hauled nonpasteurized liquid egg, which was contaminated with *S. enteritidis*. There was evidence that the trailers were not adequately cleaned after transporting the egg, and the ice cream premix was not repasteurized after transportation. Detailed analyses of samples associated with the outbreak indicated that betweeen 25 and 50 cells of *S. enteritidis* caused mild to severe illness, and that children experienced more severe symptoms than adults.[113] The total number of people thought to have been affected (about 22,400) was estimated on the basis of the attack rate in Minnesota, where the outbreak was detected, and the number of people nationwide who consumed the product during the relevant period of time.

Two outbreaks of infection with *S. enteritidis* reported in Germany in 1996 were attributed to the consumption of ice cream prepared in commercial bakeries.[110]

In an outbreak in 1987 in the UK in which six members of a family suffered food poisoning due to *Bacillus cereus* after eating homemade ice cream, the product was made with egg white and fresh cream and was reported to contain $>10^{10}$ *B. cereus* per ml.[101]

In January 1991, epidemic cholera spread to Piura in Peru and within 2 months caused 7,922 cases and 17 deaths.[88] Epidemiological studies indicated that the organism was spread in municipal drinking water and in food and beverages sold by street vendors; the use in those beverages of ice prepared in conditions of very poor hygiene was probably one of the causes of transmission.

In 1991, four cases of cholera in the US were caused by frozen coconut milk that was imported from Thailand and used to prepare a dessert.[109]

An outbreak of infection with *E. coli* O157 in 1997 in the US that affected 15 people was associated with the consumption of frozen ground meat patties and burgers.[20] These patties were not a ready-to-eat product, and an important critical control point in the production of such foods is adequate cooking before consumption in order to ensure that any surviving pathogenic microorganisms are killed.

Seventeen members of a group of travelers from Italy who had visited Thailand in 1988 showed symptomatic infection with *Entamoeba histolytica* and/or *Giardia lamblia*[61] while other members of the group were asymptomatically infected. Foods that were significantly associated with the infections were drinks with ice, ice cream, and raw fruit in ice. It was suggested that ice or ice cream was the vehicle for *Entamoeba histolytica,* whereas *Giardia lamblia* was transmitted in water used with the raw fruit and drinks.

In 1987, an outbreak of infection with Norwalk virus in the US was associated with consumption of commercial ice used in carbonated drinks.[17] The ice had been produced in an ice manufacturing plant from three wells, it was then treated with ultraviolet light before freezing. Prior to production of the ice, a torrential rainfall caused flooding of the manufacturer's wells and septic tank. Ice production was halted for 2 days and resumed after turbid water had been pumped out of the wells; the water was not chlorinated before production of ice was resumed.

Table 6-8 shows two outbreaks due to infection with hepatitis A virus. In the first, frozen raspberries were implicated and it was suspected that frozen raspberries had been implicated in several previous outbreaks.[86] The distributor blast-froze raspberries gathered from a number of farms, small holdings, and large private gardens. Berry picking was a seasonal occupation attracting large numbers of itinerant workers. Cases of illness due to hepatitis A were reported in the area at the same time that the fruit was being picked. It was presumed that the berries became contaminated during picking and/or packing, probably by a virus excreter among the pickers. The defrosted raspberries were used to prepare a mousse and, in contrast to strawberries, they were not generally washed prior to serving.

A second outbreak of hepatitis A infection occurred in the US in March 1997 and was associated with frozen strawberries.[19] The fruits had been harvested in Mexico and transported to California, where they were processed, packed, and frozen in April to May 1996.

Table 6-8 shows examples of outbreaks that have been documented and reported. It should be remembered that in many countries additional outbreaks have undoubtedly occurred that have either not been detected, or not investigated and reported.

6.8 MICROBIOLOGICAL SPOILAGE OF FROZEN FOODS

At –5°C, a frozen food containing ice in equilibrium will have an a_w of about 0.95, and at –10°C the a_w will be about 0.90. Consequently, any microorganisms capable of growing in these low temperatures in media or foods in which freezing has started must be capable of growth at low water activity. The minimum temperature at which growth of spoilage bacteria occurs is probably between –2° and –10°C, but reliable reports of growth between about –5° and –10°C were stated by Larkin and Stokes[63] to be rare. On agar media containing glycerol or glycerol plus ethylene glycol to prevent freezing two Gram-positive cocci, one Gram-negative rod, and four species of *Candida* grew at –2°C and the yeasts also grew at –4.5°C while three species of *Bacillus* grew at –7°C and two at –10°C.[63] In a liquid medium containing 10% glycerol to prevent freezing (a_w approximately 0.980) three strains of *Candida* species grew at –5° to –7°C, and five strains of *Bacillus* species grew at this temperature with generation times of 8.5 to 11.5 days. Growth of spoilage bacteria is not likely to be a problem in frozen foods maintained at temperatures of –12°C or lower, and would be very slow in foods maintained between –5° and –12°C. The presence of a high number of microorganisms on meat or fish before freezing may cause slow spoilage during frozen storage. This is due to the fact that although the microorganisms gradually die during storage of frozen foods, their enzyme systems, in particular lipases and lipoxidases, may remain active at temperatures down to –30°C.

Fish and other seafood from cold water environments may be heavily contaminated with psychrophilic and psychrotrophic bacteria. Experiments were reported to show that psychrophilic marine vibrios caused hydrolysis of protein and deterioration of quality in frozen fish and seafood stored at –20°C for 12 weeks[66]; the samples had been inoculated with the vibrios by dipping into a suspension containing 5×10^4 vibrios ml^{-1}.

Some molds are reported to grow on frozen foods at temperatures down to about –8°C.[94] Frozen meat transported for long distances by sea has been affected by a type of spoilage termed "black spot."[64] This has been attributed mainly to *Cladosporium herbarum*, but there is evidence that *Cladosporium cladosporioides*, *Aureobasidium pullulans*, and *Penicillium hirsutum* are also involved. "White spot" spoilage can occur on frozen meat and poultry and is caused mainly by *Chrysosporium pannorum*, but species of *Acremonium* and immature colonies of *Penicillium* species are reported to produce similar spoilage.[64] "Whisker-like" spoilage of meat is due mainly to *Thamnidium elegans* and occasionally to *Mucor racemosus* while the formation of blue-green colonies is due to *Penicillium* species, particularly *Penicillium expansum*. The minimum temperature for growth of these molds on meat was estimated as probably between –5° and –6°C.[64] Growth of these molds at –5°C is very slow; it was calculated that the minimum time for formation of a barely visible colony of *Cladosporium herbarum* at –5°C would be more than 4 months, and in a storage trial black spot colonies were only visible on meat after storage for 35 weeks.[64] In practical conditions it is thought that black spot, white spot, and blue-green mold develop as a result of the surface of meat reaching a temperature higher than –5°C in situations where surface dessication limits growth of spoilage bacteria. *Thamnidium elegans* is no more tolerant of low a_w than are spoilage bacteria, and "whiskers" spoilage by this mold probably occurs when temperature fluctuations lead to periodic defrosting of the surface of frozen meat and to high humidity from condensation.

On exposed muscle surfaces of frozen lamb carcasses stored for prolonged periods at –5°C, a yeast-dominated microflora was reported.[64] Yeasts were reported to multiply more rapidly than molds or bacteria on meat at –5°C, forming tiny, discrete colonies. The main yeasts present were *Cryptococcus laurentii* var. *laurentii*, *Cryptococcus infirmo-miniatus*, *Trichosporon pullulans*, and *Candida zeylanoides*. These yeasts have also been found in the microflora of turkeys stored for long periods at –2°C. Growth of a *Cryptococcus* species has been reported on frozen poultry at –5°C, and limited growth was reported at –7.5°C but not at –10°C.

6.9 CONCLUDING REMARKS

The production and use of frozen foods is likely to continue to increase. New technologies that may be applied in this production include high-pressure processing to achieve more rapid freezing and thawing throughout foods, dehydro-freezing, and use of antifreeze proteins.[119] Techniques that result in optimum quality of frozen foods tend to be those that favor the survival of bacteria, yeasts, and molds. Apart from situations in which specific conditions are used that are known to kill protozoa and other parasites, it is probably appropriate to consider that the best freezing practice, which consists of rapid freezing followed by storage at nonfluctuating, low temperature, has the least effect on microorganisms. It is safest, therefore, to assume that freezing and frozen storage do not necessarily cause a marked decrease in the microbial contamination of food.

The incidence of food poisoning associated with frozen foods is very low compared with the amount of these foods consumed. Nevertheless, in recent years a number of outbreaks associated with frozen foods, in particular with ice cream, have been documented. Some of these outbreaks have been very extensive. The increase in global production and marketing of frozen foods such as vegetables and seafoods could lead to an increase in such outbreaks unless sufficient attention is given to possible sources of contamination. Prevention of food poisoning from frozen foods depends primarily on the manufacturer, whether this is a large company or a small-scale producer or caterer. Purchasers also have a responsibility to follow the instructions of the manufacturer regarding storage and preparation. Because many types of food can be frozen a very short time after production or harvesting, freezing offers a major means of preservation of food while retaining excellent quality.

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7 Drying and Reduction of Water Activity

John H.B. Christian

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7.1 INTRODUCTION

Many foods, such as grains, nuts and other seeds, are dry at harvest and if maintained under dry conditions are immune from microbial attack and spoilage. Other foods, of normally high moisture content, have been preserved from prehistoric times by drying with the application of heat from the sun or from fire. The microbiological stability of these foods is not a function of their total moisture content but is related to the proportion of this water that can be utilized for growth by contaminating microorganisms.[185] Water activity (a_w) is generally accepted by microbiologists as the expression of available moisture that best correlates with the growth of microorganisms under conditions of reduced water content.[185] Thus, *Staphylococcus aureus* grew in dried meat at water contents down to 23.3%, in dried milk down to 15.7%, and in dried soup down to 62.5%. The levels of a_w at these very different growth-limiting water contents were 0.88, 0.86, and 0.86, respectively.

The a_w of a food may also be reduced, and its microbial stability enhanced, by the addition of solutes. This most commonly involves the addition of sodium chloride to meats, fish, and vegetables, and of sucrose to fruit and fruit products. As with the drying of foods, the effect is to increase the concentration of solutes within the food. Again,[185] 9 of 13 strains of *S. aureus* grew at a_w levels down to 0.86 in nutrient broth with added NaCl, while 4 of 13 grew only to 0.88. Six of 6 strains grew to 0.88 in the same medium adjusted to a range of a_w with sucrose.

Historically, the earliest foods preserved by reduction of a_w were probably meat or fish dried in the sun or over fires. Prior addition of salt, particularly to fish, gave some insurance against the effects of rain during sun drying. Salting by "pumping" is a common practice in the curing of meats. Grapes and stone fruit are commonly sun dried, while syruping preserves jams and jellies, and production of condensed milk involves both concentration and sucrose addition. Preserved foods of very low a_w include chocolate, noodles, biscuits, crackers, and dried egg, milk, and vegetables. The low a_w levels here are a consequence of the need for very low water contents to maintain organoleptic quality; they are well below those necessary to bestow microbial stability. Many other foods are preserved by combination treatments in which smaller reductions in a_w are combined with microbiologically adverse conditions of pH, temperature, preservative, or other factors. Current consumer demands for reduced salt and reduced energy foods, and for low-fat, high moisture foods where solutes are dissolved in more water, make a_w considerations increasingly significant.[200]

7.2 THE CONCEPT OF WATER ACTIVITY

The water activity (a_w) of a solution is the ratio of the vapor pressure of the solution (p) to the vapor pressure of pure water (p_0) at the same temperature:

$$a_w = p/p_0 \tag{1}$$

As a solution or a food becomes more concentrated, p decreases and a_w falls from near the maximum value of 1.0 for pure water (in the absence of capilliary or adsorptive forces). The a_w of the solution is thus equivalent to the relative humidity of the atmosphere in equilibrium with the solution, normally expressed as a percentage (ERH):

$$\text{ERH}(\%) = a_w \times 100 \tag{2}$$

The osmotic pressure (OP) of a solution, which presupposes the presence of a membrane of appropriate permeability properties, is related to a_w by the expression:

$$\text{OP} = -RT \log_e a_w/\bar{V} \tag{3}$$

where R is the gas constant, T is the absolute temperature, and V is the partial molal volume of water. The partial molal volume of a solute or solvent is the increase in volume produced when 1 mole of that solute is added to an infinite volume of its solution at constant temperature and pressure. For water it is $\sim$18.0 cm^3 mol^{-1}.[21]

The relationship between the concentration of an ideal solution and its vapor pressure is expressed by Raoult's Law in the form:

$$p/p_0 = n^2 / (n^1 + n^2) \tag{4}$$

where n^1 and n^2 are the number of moles of solute and solvent, respectively. Thus, here the vapor pressure of the solution relative to that of pure water is equal to the mole fraction of the solvent. One kg water contains 55.51 moles, so that for a 1 molal aqueous solution of an ideal solute:

$$p/p_0 = n^2 / (n^1 + n^2) = 55.51 / (1 + 55.51) = 0.9823$$

However, the a_w values of 1 molal solutions of sucrose and of NaCl are 0.981 and 0.967, respectively, because the solutions are not ideal. Interactions between molecules and ions reduce the effective number of solute particles, while dissociation increases the number. Experimentally derived factors, molal osmotic coefficients, may be used to compensate for nonideal conditions and to permit calculation of accurate a_w values.[177] Note that concentrations are expressed as molal (m), gram moles of solute per kg of solvent, not as Molar (M), gram moles of solute per liter of solution. A detailed discussion of water status in relation to microbiological systems is available elsewhere.[21] In the food industry, various other expressions of solute concentration are used, some specific to the solute, eg, %(w/w), %(w/v), °Brix, °Baumé, °Salinometer. Table 7-1 shows the relation of such values to a_w. Methods for determination of a_w are discussed in Section 7.10.

In fact, foods, particularly those of low and intermediate moisture, are frequently not in true equilibrium, suggesting that an expression such as a_w derived for an equilibrium situation is inappropriate for application to such foods.[195] However, in terms of the shelf life of the foods, changes in water vapor pressure resulting from this inequilibrium appear generally to be within the experimental error of determining a_w ($\sim \pm 0.01\ a_w$).[35] On the other hand, there are situations in which foods exhibit pronounced gradients in a_w, generally as a consequence of temperature gradients[219] (see Section 7.3.1).

Foods that are not in thermodynamic equilibrium may be in a metastable, amorphous state. These are very sensitive to changes in temperature and moisture content and can exist in either a "glassy" or a "rubbery" state[195] as shown in Figure 7-1. Diffusion of water and solutes would be expected to be greatly impaired by the higher viscosity in the former, which might thus be more inhibitory to microbial growth than foods in the "rubbery" state. It has been suggested[195] that the temperature, T_g, at which transition between glassy and rubbery states occurs is the parameter that can define the microbial stability and safety of foods much more reliably than can a_w. It is clear from studies on the use of various solutes to control a_w in laboratory media that there can be marked solute effects on microbial growth,[41] and that factors other than a_w are sometimes important.[79]

Table 7-1 Concentrations of NaCl and Sucrose at Various Levels of Water Activity (a_w) at 25°C

	NaCl			*Sucrose*		
a_w	*m*†	*%, w/w*	*°Salinometer*	*m*†	*%, w/w (°Brix)*	*°Baumé*
1.000	0	0	0	0	0	0
0.990	0.300	1.74	6.57	0.534	15.45	8.59
0.980	0.607	3.43	12.94	1.03	26.07	14.43
0.960	1.20	6.57	24.79	1.92	39.66	21.79
0.940	1.77	9.38	35.40	2.72	48.22	26.34
0.920	2.31	11.90	44.91	3.48	54.36	29.57
0.900	2.83	14.18	53.51	4.11	58.45	31.69
0.880	3.32	16.28	61.43	4.93	62.77	33.90
0.860	3.80	18.18	68.60	5.58	65.63	35.36
0.840	4.26	19.94	75.25			
0.820	4.71	21.59	81.47			
0.800	5.15	23.13	87.28			
0.753	6.16	26.5	100.00			

†Molality, gram moles per kg of water.

Source: Data from International Commission on Microbiological Specifications for Foods, Factors Affecting the Life and Death of Microorganisms, in *Reduced Water Activity in Microbial Ecology of Foods*, Vol. I, © 1980, Academic Press and from R.A. Robinson and R.H. Stokes, *Electrolyte Solutions*, © 1959, Butterworth-Heineman.

To date, there is little experimental evidence to support the proposition that glass transition temperatures predict microbial stability, although the concept that viscosity could play an important role is an attractive one. Certainly, the moisture content of foods can profoundly affect the glass transition temperature.[194] The microbiological evidence that has been presented[195] appears to have been refuted.[34] Further, recalculations of published data on the water relations of microbial growth in terms of glass transitions in food systems did not appear to support the contention that mobility considerations allowed accurate prediction of microbial stability.[35] However, "the use of state diagrams, indicating material physical state, combined with water activity effects on processability and deterioration, increases the validity of food stability and shelf-life predictions."[178]

The anomolous results obtained in microbiological growth experiments, referred to as "specific solute effects" (see Section 7.8), do not appear to be well explained by invoking phase transitions, although these may be important in particular cases. Because many foods are not in thermodynamic equilibrium, a_w is an inappropriate term to apply to them.[167] However, since a "pseudo equilibrium" may exist in such foods (and persist for longer than the shelf life of the food), the "pseudo water activity" that is measured will in many cases be very close to a true thermodynamic equilibrium, a_w.[225] Until a more appropriate title is given to the ratio of the measured water vapor pressure in food, whether in thermodynamic equilibrium or not, to the vapor pressure of pure water, "water activity" will probably continue to be the preferred term in food microbiology.

7.3 WATER ACTIVITY AND THE WATER CONTENT OF FOODS

For foods high in soluble components, accurate estimates of a_w can be made from knowledge of water and solute contents.[35] However, the water activity of a food as a function of its water content is more commonly determined by construction of a water sorption isotherm.[71] This is a graph of a_w against a range of water contents at constant temperature (Figure 7-2). (See Section 7.10 for determination of a_w). Instructions for the standardization of isotherm measurements are given elsewhere.[239] The sorption isotherm is generally sigmoid in shape and three regions may often be identified. In region A, water is strongly absorbed to the food as a monolayer. The water in region B is less strongly held in additional hydrogen bonded layers and in small capillaries. In region C, the water is condensed in larger capillaries or other larger spaces or is free in solution. For math-

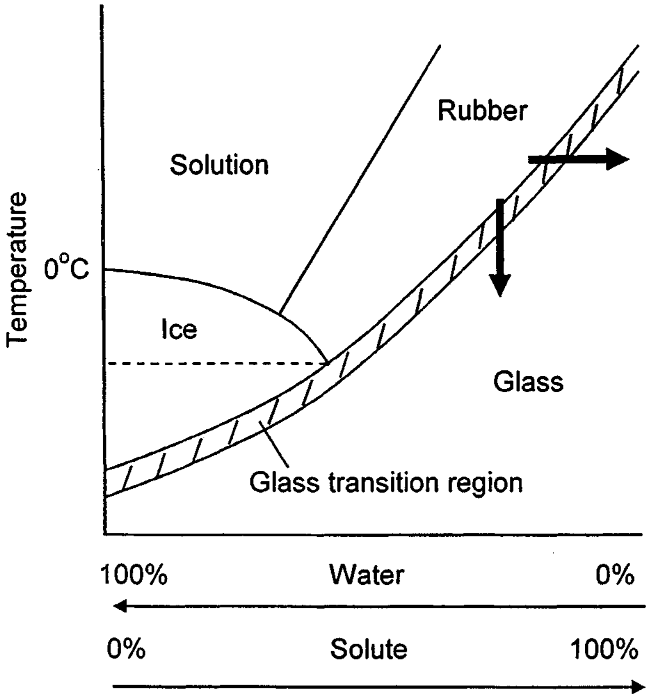

Figure 7-1 The Relationship of the Glass Transition Region to Solute Concentration and Temperature. Viscosity rises sharply through the glass transition region in the directions of the broad arrows. Copyright © 1988, G.W. Gould and J.H.B. Christian.

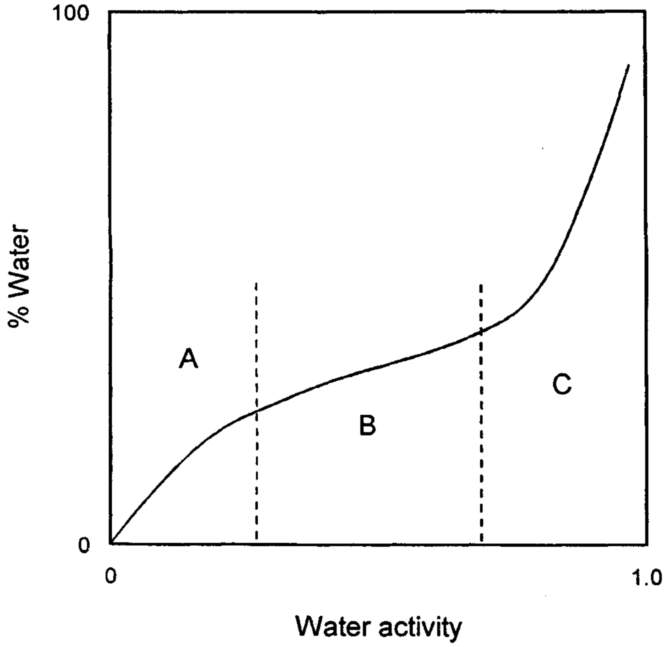

Figure 7-2 Generalized Water Sorption Isotherm. For explanation of A, B, and C, see text. *Source:* Adapted with permission from T.P. Labuza, Sorption Phenomena in Foods, *Food Technology*, Vol. 22, pp. 262–272, © 1968, Institute of Food Technologists.

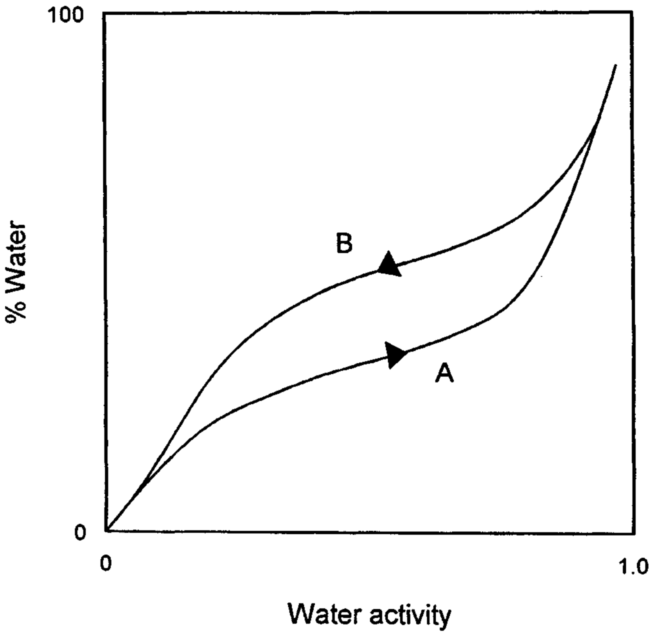

Figure 7-3 Water Sorption Isotherms Showing Hysteresis Loop. Curve **A**, absorption isotherm; curve **B**, desorption isotherm. *Source:* Adapted with permission from T.P. Labuza, Sorption Phenomena in Foods, *Food Technology*, Vol. 22, pp. 262–272, © 1968, Institute of Food Technologists.

ematical models that have been developed to relate a_w to sorbed water, see Rahman.[164]

Isotherms obtained by removal of water from a moist food and by addition of water to a dry food may differ (hysteresis), with water contents in central parts of the desorption curve being higher than in the absorption curve (Figure 7-3).[103,111] No quantitative prediction of hysteresis has been reported.[164] Theories to explain why a much lower vapor pressure is needed to reach a given water content by desorption than by absorption have been based on the structure of the sorbent. They include capillary condensation, partial chemisorption, structural changes in nonrigid solids, and polar sorption in biological materials.[103]

Hysteresis may be expected to have consequences for the microbial stability of foods. However, in foods showing marked hysteresis effects at reduced a_w levels produced by desorption, microbial growth responses were similar to those obtained in laboratory media.[113] When the a_w levels were adjusted by adsorption, inhibition occurred at higher values. These experiments suggest that there is a "fail-safe" aspect to the extrapolation to foods of data obtained in laboratory media. The approximate a_w values of a range of foods are given in Table 7-2. These are in-

Table 7-2 Approximate Water Activity Values for Selected Foods at "Normal" Moisture Contents

Range of a_w	*Foods*
1.00–0.95	Fresh foods (meat, fish, milk, eggs, fruit, vegetables), bread, liver sausage, Bologna sausage, canned fruit in syrup, canned vegetables in brine, low-salt bacon, processed cheese
0.95–0.90	Raw ham, salami, bacon, some hard cheeses, mayonnaise, low-calorie jams
0.90–0.85	Aged hard cheeses, dry ham, fruit juice concentrates, maple syrup (saturated sucrose solution; 0.86 a_w)
0.85–0.80	Sweetened condensed milk, parmesan cheese, fruit cake, high moisture prunes
0.80–0.75	Heavily salted fish, molasses, jams, conserves, marzipan, glacé fruits, prunes, fondants (saturated NaCl solution; 0.75 a_w)
0.75–0.65	Some dried fruit (dates, figs, sultanas) and nuts, dendeng, rolled oats, malt extract
0.65–0.60	Some dried fruits, honey, some confectionery
Below 0.60	Dry pasta, spices, biscuits, crackers, dried milk, dried egg, dried vegetables, flour, instant coffee, cereals

Source: Copyright © 1978, J.A. Troller and J.H.B. Christian.

dicative only, as published values vary greatly, because of the large variations in the moisture contents of some foods.

7.3.1 Influence of Temperature

Plots of water content against a_w are termed "isotherms," indicating that all measurements are made at the same temperature. Temperature affects a_w through its influence on water vapor pressure, as indicated in the tables in Resnik and Chirife.[168] The effects are small within the microbial growth range, unless the solution is saturated, when temperature will influence the amount of solute in solution. In a frozen system at equilibrium, the vapor pressures of solid water (as ice crystals) and of the interstitial concentrated solution are identical.[63] Thus, at temperatures below the freezing point of a solution or a food, a_w depends only on temperature.[187] A food at -5°C has an a_w of 0.953, and a food at -20°C has an a_w of 0.823, irrespective of composition.[187] In these cases, a_w is the ratio of the vapor pressure of ice to the vapor pressure of liquid (super cooled) water at the same temperature. Clearly, a_w as well as temperature may have a bearing on microbial growth in foods held at relatively high frozen temperatures.

Temperature gradients within foods can be important where microbiological stability depends on a_w. Water will migrate as water vapor from warmer to colder areas, with consequent increase in a_w in the colder regions. Such migrations may result from diurnal temperature variations and can be of major mycological significance in stored commodities such as grains.[232] Migrations can also be observed within packaged foods in retail chill cabinets where marked temperature gradients may exist, even leading to visible condensation of pure water on food surfaces or the inner surfaces of packaging materials.

7.4 WATER RELATIONS OF MICROBIAL GROWTH

The optimum a_w for the growth of the majority of microorganisms is in the range 0.995–0.980. As a_w is reduced below this optimum, growth rate declines, often linearly; stationary population falls after an a_w range of relative constancy and the duration of the lag phase increases[185] (Figure 7-4). Growth ceases at the a_w where lag becomes infinite. As experiments

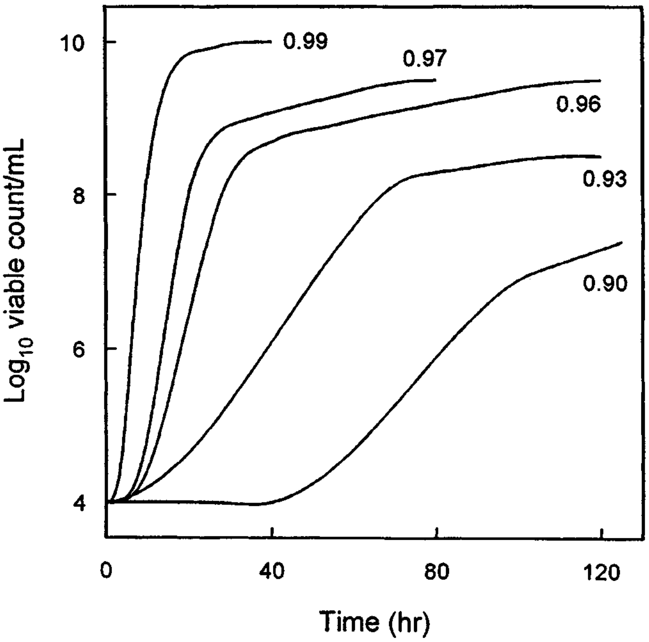

Figure 7-4 Growth Curves of *Staphylococcus aureus* in Laboratory Medium Adjusted to Various Levels of a_w with Hydrolyzed Protein. *Source:* Adapted with permission from J.A. Troller, Effect of Water Activity on Enterotoxin B Production and Growth of *Staphylococcus Aureus*, *Journal of Applied Microbiology*, Vol. 21, pp. 435–439, © 1971, American Society for Microbiology.

are never infinite, the minimum a_w permitting growth is the lowest a_w at which growth was observed during an experiment, and it is therefore important that the duration of such experiments be reported. The most important of these cardinal values, in terms of the microbiological safety and quality of foods, is the minimum a_w for growth. For most bacteria and fungi, growth is confined to the a_w range above 0.90, but in both groups there are organisms of great importance to food preservation and safety that grow at substantially lower levels of a_w. Microorganisms capable of growth below 0.85 a_w are termed xerophiles,[154] and may include groups also described as osmophiles and halophiles. Note that, while both bacteria and fungi have representatives that can grow in highly saline environments, only yeasts and filamentous fungi have strains that can multiply in very high concentrations of sugar. Growth of bacteria and fungi at reduced a_w in specific foods is discussed in Section 7.9.

Most data on the minimum levels of a_w permitting growth of microorganisms are obtained in laboratory media where a_w can be defined to at least 0.002 units.[185] This contrasts with the situation in foods where the accuracy of a_w measurement may be ±0.02 a_w units (see Section 7.10). Accurate comparison of growth limits in media and in foods is therefore difficult.

7.4.1 Bacteria

In general, bacteria grow more rapidly than fungi, so that in conditions favorable to both, bacteria will usually become dominant. The optimum a_w for growth of most species is lower than the a_w of some standard media. Thus, addition of solute to nutrient broth to reduce its a_w from 0.999 to 0.995 will increase the rate of growth of *Escherichia coli* and *Streptococcus faecalis*,[234] *Salmonella oranienburg*,[44] and *Staphylococcus aureus*[185] by about 10%. The a_w of basal brain heart broth is lower at 0.993.[42] Further reduction in a_w results in a decrease in growth rate that is often, but by no means always, linear. This decrease is very steep for organisms with high water requirements and much less so for those capable of growth in physiologically drier conditions. In contrast, the stationary population may remain high until an a_w is reached, which reduces growth rate by half.[185] Lag phase is usually assumed to be shortest where growth is most rapid. This appears to be true in water relations studies. As lag lengthens with reduction in a_w, viability often decreases markedly, eg, by 90%,[75] before multiplication commences, and a very long period may ensue before the original inoculation level is reached.

With spore-forming bacteria, particularly in foods that have received a mild heat treatment, a prerequisite for growth may be spore germination. However, spores of some species may germinate at a_w levels that are too low for outgrowth and growth to proceed. This is true of types A, B, and E of *Clostridium botulinum*[9] and for some *Bacillus* species.[77] However, for *Bacillus cereus*, minimum a_w levels for germination and outgrowth were similar when a_w was adjusted with NaCl.[94]

Traditionally, most emphasis has been placed on the lowering of a_w required to prevent growth of a particular organism. Strangely, this is most often expressed in terms of the lowest a_w permitting growth. The relation of this value to the highest a_w at which growth is completely prevented depends upon the increments of a_w employed in establishing the lower limit. Hence, the lower limit and the level of a_w preventing growth given in the literature are often not very precisely determined. In the practice of food preservation, the a_w required to control growth of a particular microorganism is either determined accurately in the relevant food by a challenge test, or a substantial margin of safety is allowed in utilizing published data.

Table 7-3 shows approximate minimum a_w values for the growth of a range of bacteria of relevance in

Table 7-3 Approximate Minimum Water Activity Values for Growth of Bacteria in Salt-Adjusted Laboratory Medium

Bacteria	*Minimum a_w*	*Reference*
Aeromonas hydrophila	0.97	151
Bacillus cereus	0.93	130
Bacillus stearothermophilus	0.93	41
Campylobacter jejuni	0.98	56
Clostridium botulinum type A	0.94	9
Clostridium botulinum type B	0.94	147
Clostridium botulinum type E	0.97	147
Clostridium perfringens	0.97	101
Escherichia coli	0.95	130
Halobacterium halobium	0.75	24
Lactobacillus helveticus	0.95	182
Listeria monocytogenes	0.92	211
Micrococcus halodenitrificans	0.85	13
Pseudomonas fluorescens	0.97	130
Salmonella species	0.95	44
Staphylococcus aureus	0.86	185
Streptococcus lactis	0.97	182
Vibrio costicola	0.85	13
Vibrio parahaemolyticus	0.95	16

foods. All values were determined with a_w adjusted with NaCl and with other environmental factors, such as temperature and pH, close to optimal for growth. The a_w values have been rounded off to two decimal places, as the methods used to determine a_w are likely to be accurate to ±0.005 a_w units (see Section 7.10). Some generalizations can be made. Pseudomonadaceae have high water requirements, with some strains having lower limits of about 0.97 a_w. Most Enterobacteriaceae, lactic acid bacteria, and clostridia grow down to 0.94 a_w. Some aerobic spore-forming bacteria cease to grow at about 0.90 a_w, with Gram-positive cocci even more resistant to physiological drought. Below about 0.85 a_w, the only bacteria that multiply are halophilic, some of which can grow in media or food saturated with respect to NaCl (0.75 a_w).

Foodborne Bacterial Pathogens

All of the foodborne pathogenic bacteria will grow at a_w of 0.98 or above. This is the range in which are found fresh foods such as fruit, vegetables, meats, fish, and milk. *Campylobacter jejuni* is the most a_w-sensitive foodborne pathogen, with a lower limit of about 0.98 a_w, with growth in 1.5% NaCl but not in 2.0%.[56] As it requires temperatures above 30°C for growth, dies rapidly at 48°C, is microaerophilic, and requires enhanced CO_2 concentration,[204] it could multiply in foods only under quite specific environmental conditions.

More tolerant of reduced a_w are the nonproteolytic (Group II) strains of *Clostridium botulinum* (types E, F, and nonproteolytic B), with a minimum a_w for growth of 0.97 (5% NaCl).[9,132,147] Toxin was produced at all a_w levels at which growth occurred.[147] As the minimum temperature for growth of Group II strains is approximately 3.5°C[184] and their spores are of low heat resistance[85] (see Chapter 41), the greatest hazard of botulism caused by these strains is in inadequately heated, lightly salted foods, particularly fish products (see Chapter 21). Spores of type E are the most commonly isolated *C. botulinum* from both marine and fresh waters and sediments.[85] *Aeromonas hydrophila*[151] and *Pleisomonas shigelloides*[107] are of similar salt tolerance to Group II *C. botulinum.*

The largest group of foodborne pathogenic bacteria has minimum a_w for growth in the range of 0.95 to 0.94. Included are members of the Enterobacteriaceae. Fifteen motile serotypes of *Salmonella* showed very similar water relations for growth in liquid laboratory media, with limiting a_w of 0.95–0.94.[44] A nonmotile strain of *S. enteriditis* was more sensitive. Nonpathogenic[219] and pathogenic[74] *Escherichia coli* also have lower limits of about 0.95 a_w. One of the few halophilic foodborne pathogens is *Vibrio parahaemolyticus*, requiring at least 0.5% NaCl for growth. The minimum a_w for growth in salt-adjusted medium is 0.95.[16] It is a common contaminant of seafoods.

Spore formers are well represented in this group. *Bacillus cereus*, a common contaminant of cereals and milk, grows to a minimum a_w of about 0.95.[163] However, as with other *Bacillus* species, some properties of *B. cereus* are subject to wide variation.[108] Psychrotrophic strains, capable of growth at 5°C, have been isolated from milk,[192] and most of the psychrotrophic strains tested produced the diarrheal enterotoxin.[80] The maximum salt concentration permitting growth of Group I *Clostridium botulinum* types A and proteolytic B was about 10% NaCl (0.94 a_w).[85] However, with a_w adjusted with a three-salt mixture, lower limits for the two types differed, being 0.95 for type A and 0.94 for type B.[147] Toxin was produced at all a_w levels at which growth occurred, although sometimes of low titre.[147] *Clostridium perfringens* also has a lower limit for growth in NaCl-adjusted medium of 0.95 a_w.[101]

Listeria monocytogenes is one of the foodborne pathogens best able to multiply at reduced a_w. Its lower limit is 0.92 a_w in NaCl.[50,152,211] It is also psychrotrophic and ubiquitous, and of particular concern in chilled foods. The only bacterial pathogen with lower water requirement, and the most studied in this context, is *Staphylococcus aureus*. Nine of 13 strains of *S. aureus* grew at a_w levels down to 0.86 in nutrient broth with added NaCl; the other 4 strains grew to 0.88 a_w.[185] The lower limit of 0.86 a_w has been confirmed in extensive studies,[7,61] although growth has been reported at 0.83 a_w.[212] Growth in reconstituted dried foods was also prevented below 0.86 a_w.[185] Although a facultative anaerobe, *S. aureus* grew only down to 0.92 a_w under anaerobic conditions.[185] Enterotoxin A and D production by *S. aureus* occurred at nearly all levels of a_w permitting growth,[61,145] but synthesis of enterotoxins B and C was much more sensitive, being prevented in the a_w range 0.96–0.93.[145,217]

Halophilic Bacteria

In addition to tolerating high levels of salt, and hence growing at low a_w, halophiles are unable to grow at very high a_w. The lowered a_w required for growth must be provided by inorganic salts, with NaCl predominating.[219] The widely studied halophiles have been classed as moderate and extreme.

Moderate halophiles require 0.2–0.5 M NaCl (1.2% to 2.9% w/v) for growth (~0.99–0.98 a_w). They grow optimally at about 1 M NaCl (5.9% w/v) (~0.96 a_w), with growth ceasing at 3.5–4.0 M NaCl (20% to 23% w/v) (~0.85 a_w).[13,110] The extreme, or red obligate, halophiles, which are archaebacteria, include the genera *Halobacterium* and *Halococcus*. Growth requires about 3 M NaCl (17.5% w/v) (~0.88 a_w), is optimal at about 4 M (23% w/v) (0.85 a_w), and continues to saturated salt (0.75 a_w).[24,25,110] Although it does not appear to have been explored, it is possible that growth would proceed at even lower a_w if a suitable medium were devised combining high concentrations of NaCl with other solutes. This is likely because at a_w levels below that necessary to prevent lysis, there was little change in growth rate of extreme halophiles with further addition of NaCl.[33] Similarly, in saturated salt medium, *Halobacterium halobium* multiplied only 25% slower than at its optimal salt concentration.[93] Moderate halophiles have been the major spoilage agents of meat curing brines[196] and the extreme halophiles the bacterial spoilage agents of salted, dried fish.[15] Halophiles grown in high-salt environments lyse readily on dilution,[43] so that hosing contaminated plant and equipment with fresh water gives some measure of control of these organisms.

7.4.2 Fungi

Yeasts and molds are generally found in nature in more acid or drier environments than bacteria. Thus many, but by no means all, are more tolerant of reduced a_w than most bacteria, and lower levels of a_w are required to prevent their growth.[4] Pitt's definition of xerophiles as microorganisms capable of growth under at least one set of environmental conditions at a_w below 0.85 includes those fungi that have been termed osmophiles and halophiles.[154] Most such organisms are fungi. The minimum a_w levels permitting the growth of a range of fungi relevant to foods are shown in Table 7-4.

The lowest a_w at which growth of bacteria has been recorded is 0.75 (saturated NaCl). In contrast, the most xerophilic fungi can grow at 0.62–0.61 a_w in the presence of high concentrations of sugars.[124,155,229] Not only are such organisms growing in remarkably high concentrations of solutes, but the replication of genetic material is taking place close to 0.55 a_w, the a_w at about which DNA becomes disordered.[62] Most of the organisms capable of growth below 0.90 a_w are ascomycetous fungi or conidial fungi.[159]

Table 7-4 Approximate Minimum Water Activity Values for Growth of Food-Related Fungi

Molds	*Minimum a_w*	*Reference*
Aspergillus flavus	0.80	160
Aspergillus ochraceus	0.77	155
Aspergillus parasiticus	0.80	160
Aspergilus penicillioides	0.73	159
Aspergillus restrictus	0.71	198
Botrytis cinerea	0.93	199
Byssochlamys nivea	0.84	150
Chrysosporium fastidium	0.69	155
Chrysosporium xerophilum	0.71	155
Eremascus albus	0.70	186
Eurotium amstelodami	0.70	159
Eurotium chevalieri	0.74	5
Eurotium echinulatum	0.62	199
Eurotium rubrum	0.70	199
Paecilomyces variotii	0.84	155
Penicillium chrysogenum	0.79	155
Penicillium cyclopium	0.82	5
Penicillium expansum	0.83	136
Penicillium islandicum	0.83	5
Penicillium patulum	0.81	150
Penicillium viridicatum	0.81	136
Polypaecilum pisce	0.71	237
Rhizopus stolonifer	0.89	159
Stachybotrys atra	0.94	96
Wallemia sebi	0.69	157
Xeromyces bisporus	0.61	155

Yeasts	*Minimum a_w*	*Reference*
Debaryomyces hansenii	0.84	138
Saccharomyces cerevisiae	0.90	41
Zygosaccharomyces bailii	0.80	161
Zygosaccharomyces rouxii	0.62	229

Of major interest are *Aspergillus flavus* and *A. parasiticus*, producers of aflatoxins. They have very similar water relations.[160] Aflatoxins are the mycotoxins that appear to be of most concern in human diets. These fungi have a predilection for nuts and oilseeds, most importantly peanuts, corn, and cottonseed.[159] While the minimum a_w for growth is about 0.78,[5] aflatoxin was not synthesized in inoculated peanuts at 0.83 a_w.[55] In corn at 30°C, aflatoxin was produced by *A. parasiticus* at 0.87 a_w.[137] A study of *A. flavus* and three related species yielded a model that predicted growth rates at a_w within the tested range of 0.995 to 0.81.[72] All the xerophilic species of *Penicillium* have lower limits for growth close to 0.80 a_w (Table 7-4). No bacteria other than extreme halophiles multiply at this a_w. An important property of many xerophilic *Penicillium* species is that they are also psychrotrophic.[159] They are the most common spoilage molds on fermented sausage and cured meats[119] and the most frequently isolated fungi from wheat in cooler climates.[232]

The large group of xerophilic *Aspergillus* spp. have lower a_w limits for growth in the approximate range 0.78–0.71 (Table 7-4). They are among the most common contaminants of grains, nuts, and spices.[88] *A. restrictus* and *A. penicillioides*, both growing below 0.75 a_w, appear to be pioneer species in the colonization of stored grains (A. Hocking, pers. comm.). The more xerotolerant group described in earlier literature as the *Aspergillus glaucus* group are now recorded as the teleomorph *Eurotium* species. Described as the epitome of food spoilage fungi,[159] members of these genera tolerate, or thrive in, elevated temperatures and reduced a_w. Growing poorly or not at all in basal media of high a_w, *Eurotium* species grow optimally at 0.95–0.94 a_w, with lower limits of 0.74–0.62.[159,186]

The other xerophilic molds listed in Table 7-4 are all important spoilage agents of dried and concentrated foods[1] including fish.[158] Of particular interest is *Xeromyces bisporus*, the most xerophilic microorganism recorded. With a minimum a_w of 0.61,[155] its ability to germinate and grow at low a_w far exceeds that of other xerophilic molds.[157]

The most important genera of xerophilic yeasts with respect to food spoilage and food fermentations are *Debaryomyces, Hansenula, Pichia, Zygosaccharomyces*, and *Torulopsis*.[89] All are reported to contain highly salt-tolerant species,[149] but none multiply below about 0.80 a_w in salted foods whereas bacterial halophiles will multiply at 0.75 a_w in NaCl. This salt-tolerant group includes strains of *Z. rouxii*, which contributes to the flavor of soy sauce,[149] yet other strains of this species are highly sugar tolerant. The lowest a_w recorded for yeast growth was 0.62 for *Z. rouxii* in a fructose syrup and a pear concentrate.[229] Another very important xerophilic yeast is *Z. bailii*, which not only grows at 0.80 a_w but also is highly resistant to preservatives.[161]

A prerequisite for fungal growth is frequently spore germination, with sporulation a consequence of growth. The water relations of these processes may be different from those of growth. Spore germination at low a_w may not be followed by growth, particularly if other environmental factors such as temperature are unfavorable.[5] For a group of xerophilic fungi isolated from salt fish,[226] the minimum a_w for growth was generally 0.02 a_w above that for germination. Germination is thus not a consistently reliable indicator of subsequent growth. The germination time of a fungal spore can be more important to the shelf life of a food than the subsequent rate of mycelial growth.[186] There is less information about the influence of a_w on sporulation, but the formation of different types of spores may show different responses to a_w. *Eurotium* species and *Xeromyces bisporus* can form the anamorphic state at an a_w level lower than that at which the teleomorph is formed.[155] For most species of *Eurotium*, the difference is about 0.02 a_w, but can be as large as 0.09 a_w units. *Xeromyces bisporus* formed cleistothecia and ascospores at a_w down to 0.67, and aleuroconidia down to 0.62 a_w.[155]

7.5 WATER RELATIONS OF MICROBIAL DEATH AND SURVIVAL

While many food processes, including heating, aim at the destruction of microorganisms in foods, others, such as fermentations, depend upon their survival. Water activity can play a major role in both destruction and preservation. Generally, heat resistance, or survival, increases as a_w is reduced from that of water (1.0 a_w). The inactivation rate of single celled organisms is essentially exponential,[206] and the rate of destruction is determined as the decimal reduction time, or *D*-value, being the time in minutes required to destroy 90% of the microbial population.

7.5.1 Bacteria

Bacterial spores provide the clearest evidence that heat resistance increases with decrease in a_w from 1.0 to a maximum in the range 0.4–0.2, and then some decrease as a_w approaches zero.[140] Not only does reduction in a_w from 0.998 to 0.4–0.2 greatly increase heat resistance, it causes the greatest increase in the normally least resistant spores. In systems where a_w was controlled by vapor phase equilibrium, this a_w reduction increased the *D*-value at 110°C for *Clostridium botulinum* type E spores by 10^5-fold. At 0.998 a_w, the difference in *D*-value between the least and most resistant bacterial spores was 10^4-fold, but at 0.4–0.2 a_w this difference had been reduced to 10-fold.[140] There are obvious implications here for the heat processing of low a_w foods.

Similar results (optimal survival near 0.3 a_w) were obtained using water vapor equilibration, and glycerol and LiCl solutions to provide controlled a_w.[84] However, the levels of resistance differed greatly among treatments. At this a_w, the maximum survival of water vapor-adjusted spores of *Bacillus subtilis* was 10 times that in glycerol, which was 10 times that in LiCl.

The most studied vegetative foodborne pathogens in relation to heat resistance and a_w are the salmonellae. Again, resistance increased with decrease in a_w, but the influence of the solute used to adjust a_w

was very marked.[51] Using a series of polyols and sugars to control a_w, it was demonstrated that, while the relationship between log *D*-value and a_w was nonlinear, that between log *D*-value and solute concentration was linear, except when glycerol was the bulk solute. It was speculated from a study of electron micrographs that glycerol, which entered the cell, was more protective against heat than the cell water alone, and that plasmolysis of the cell by other solutes gave even greater protection. When heat-sensitive and heat-resistant strains of salmonellae were heated in broth and in broth plus sucrose, NaCl, or glycerol, the changes in heat resistance could not be directly related to the a_w of the heating menstruum.[8] Clearly, any relationship between survival at high temperatures and a_w is much less obvious than that between growth and a_w. The same is true of survival of irradiation. NaCl, but not sucrose, provided some protection to *Salmonella typhimurium* on irradiated pork and chicken.[214]

It is not only the a_w of the heating menstruum that influences heat resistance. *Listeria monocytogenes* showed greatly increased resistance when permitted to adapt to a lower a_w prior to heating.[49,100] This has particular consequences for the safety of foods in respect of pathogens such as *L. monocytogenes* and *Staphylococcus aureus*, which are capable of growth at relatively low a_w levels.

Survival of microorganisms at moderate, near ambient temperatures is also influenced by a_w. In dried milk, vegetative bacteria survived longest at 0.05–0.20 a_w.[86] Thus, the a_w levels that best retain the organoleptic properties of dried foods are also those at which contaminating bacteria, including pathogens, will survive longest. Survival of bacterial spores in the dried milk was little affected at a_w down to 0.05, but fell substantially at 0.0 a_w.[86] In foods of near neutral pH, maximum survival of both *Staphylococcus aureus* and *Salmonella newport* was recorded at 0.11 a_w in air and 0.0 a_w in vacuum.[45] Salmonellae survived the production of both rubbery and glassy states in gelatin.[18] The a_w in glassy states was 0.45–0.28 and in rubbery states was 0.93–0.96. The cells were unable to multiply but remained viable, even when gelatin sheets were transferred between glassy and rubbery states. Survival was lower at a_w between 0.55 and 0.74.

As the a_w of a frozen food is a function not of the overall solute concentration but of the temperature (see Section 7.3.1), the effects of a_w and temperature on the survival of microorganisms in frozen foods cannot be separated.[187] Survival of bacterial spores was generally very high at any temperature of frozen storage, while *Escherichia coli* died quite rapidly at –2°C but remained viable at –20°C.[83] Thus, as with dried foods, frozen storage under conditions that optimize food quality may maximize survival of bacterial contaminants.

7.5.2 Fungi

The most heat-resistant fungal spores are much less resistant than most bacterial spores, but in some the heat resistance is high enough to cause major problems in processed acidic foods. These do not receive as severe a heating as nonacid products. The ascospores of filamentous fungi are more heat resistant than their conidia,[156] while the ascospores and vegetative cells of yeasts appear to possess heat resistance broadly similar to that of fungal conidia.[162] The most significant fungal spores in heat processed foods are those of *Byssochlamys* spp., with *D*-values in the range 1–12 minutes at 90°C.[14] Ascospores of *Neosartorya fischeri* are almost as heat resistant.[201]

The effect of reduced a_w on fungal spores is qualitatively similar to that on bacterial spores. Ascospores of *Byssochlamys fulva* became more resistant as a_w was reduced from 1.0 toward zero,[126] but even under dry conditions resistance was one or two orders of magnitude less than that of the most heat-resistant bacterial spores.

As noted earlier, a_w at temperatures below the freezing point is a function not of solute concentration but of temperature. Nevertheless, solute concentration can have a marked effect on fungal survival at such temperatures. *Torula* species frozen at –17.8°C in orange juice retained near 100% viability with 40% added sucrose, while viability fell appreciably in its absence.[91] Yeasts suspended in 65° Brix orange concentrate were more tolerant of freezing than cells suspended in 45° Brix concentrate.[139] Many substances produced by fungi under stress appear to provide some protection from freezing. In most cases, these are recognized osmoregulants.[99]

7.5.3 Viruses

The effect of food composition in respect to a_w on the heat resistance of enteroviruses has not been studied extensively, but it is known that some salts substantially increase their heat resistance. Enteroviruses are stabilized by 1 M $MgCl_2$ at 50°C[233]; 2 M NaCl has a similar effect,[183] even though it reduces stability at 37°C and below. Whether these are a_w-related effects is not known. As Norwalk virus is not yet culturable, little is known of its survival characteristics. However, outbreaks traced to the consumption of frozen shellfish indicate that it survives freezing in a high a_w food.

7.6 PHYSIOLOGICAL BASIS OF TOLERANCE TO LOW WATER ACTIVITY

7.6.1 Bacteria

Bacteria that can multiply in a defined minimal medium are more tolerant of reduced a_w when growing in a complex medium. *Salmonella oranienburg* could not grow at a_w below 0.97 in minimal medium unless proline was added.[39] In the presence of proline, other amino acids could be utilized and growth proceeded at 0.96 a_w. Addition of several vitamins permitted growth at 0.95 a_w.[39] Reduced a_w stimulated the uptake of potassium[59] and proline[20] in *Escherichia coli*, and similarly *S. oranienburg* accumulated potassium and proline from the medium when adjusting (osmoregulating) to a reduced a_w environment.[42] Similarly, the concentrations of potassium and amino acids within cells of *Staphylococcus aureus* increased as the a_w of the growth medium decreased.[46] Such accumulated potassium and amino acids were termed *compatible solutes*[22] and were subsequently defined as "solutes which, at high concentrations, allow all essential cell processes to function efficiently."[21] A range of amino acids and related compounds serve as compatible solutes in bacteria, including proline, glutamic acid, α-amino butyric acid,[134] and glycine betaine.[193] Trehalose is involved in osmoregulation in *Salmonella manhattan*[57] and in *Escherichia coli*.[116]

The observation that overproduction of proline by a mutant of *Salmonella typhimurium* enhanced its salt tolerance[52] led to studies of the genetics of osmoregulation. In nonhalophilic bacteria, these studies have mostly involved *Salmonella typhimurium*[28,29,97] and *Escherichia coli*.[127,165,207] The major compatible solutes accumulated, either by synthesis or transport, by the enteric bacteria are betaine, proline, and trehalose.[19] Intracellular potassium glutamate is believed to be a trigger for the induction of the genetic systems involved in osmoregulation.[19]

The extremely halophilic bacteria (*Halobacterium* and *Halococcus*) not only have an impressive tolerance of NaCl, but also have a very high salt requirement for growth.[110] In the absence of sufficient NaCl, the cell wall disaggregates.[115] To grow in this highly saline environment, the cell excludes sodium and concentrates potassium to very high levels as the main compatible solute.[23,47] Consequently, the enzymes of such bacteria are highly tolerant of potassium ions, of sodium ions, or of both, depending upon their disposition inside, outside, or within the cell membrane.[13]

7.6.2 Fungi

Among the microfungi, the osmoregulatory picture is quite different. High concentrations of electrolytes and nitrogenous compounds are not as prominent in foodborne fungi, although the former may be important in the early stages of adaptation to concentrated environments.[21] Trehalose, glycerol, and other acyclic polyols play major roles. The nonxerotolerant yeast, *Saccharomyces cerevisiae*, and the xerotolerant species, *Zygosaccharomyces rouxii*, both accumulate glycerol, but it appears[21] that in *S. cerevisiae* the process is much less energy efficient. Thus, at low levels of a_w, the metabolic balance is disturbed to the extent that growth is no longer possible. In the very salt-tolerant yeast, *Debaryomyces hansenii*, potassium is accumulated in preference to sodium[143] and, also as with halophilic bacteria, its enzymes are unusually tolerant to sodium and potassium.[2] However, the main solute concentrated during its growth at low a_w is again glycerol. Although mannitol is the polyol most commonly occurring in the fungi, its concentration decreases as the a_w is reduced. The solute commonly accumulated to high concentrations during growth of yeasts at low a_w levels is glycerol.[21]

Compatible, osmoregulatory solutes have been much less studied in the filamentous fungi, possibly because accurate estimation of extracellular water and hence of intracellular water and solute concentration is difficult. *Chrysosporium fastidium* and *Penicillium chrysogenum*, both xerophilic fungi, accumulated glycerol to high levels during growth at reduced a_w in glucose-adjusted media, while the latter also accumulated glycerol when a_w was reduced by addition of KCl.[125] The accumulation of internal solutes by four other xerophilic fungi (*Penicillium janczewskii*, *Eurotium chevalieri*, *Xeromyces bisporus*, and *Wallemia sebi*) and by one nonxerophilic fungus (*Penicillium digitatum*) has been studied by natural-abundance ^{13}C nuclear magnetic resonance spectroscopy.[90] All accumulated increasing glycerol concentrations as a_w was reduced in sorbitol- or in glucose/fructose-adjusted media, but when grown in media adjusted with NaCl only *Wallemia sebi* maintained a high internal level of glycerol at 0.85 a_w or below.[90]

In summary,[89] the accumulation of glycerol permits growth below 0.75 a_w, as demonstrated by the xerophilic yeasts and molds. Halophilic bacteria, concentrating potassium, are a special case. Accumulation by nonhalophilic bacteria of betaine, alone or with glutamate or potassium, is associated with growth to about 0.90, while accumulation of proline and/or glutamate allows growth of bacteria to 0.94–0.96 a_w. The genetic control of osmoregulation is better understood in nonhalophilic bacteria and in yeasts than in halophilic bacteria and filamentous fungi.

7.7 INTERACTIONS OF a_w WITH OTHER ENVIRONMENTAL FACTORS

Data on the minimum a_w permitting growth of microorganisms are usually, and correctly, reported for situations where other environmental conditions are close to optimal. However, except in foods of a_w below about 0.70, the use of a_w for preservation will be in combination with other factors, which will themselves usually exert an inhibitory effect. The interactions between these factors are complex and usually appear to be additive.[219] The most important in this context are the other intrinsic factors, pH, Eh, and chemical preservatives, and the extrinsic factors, high and low temperatures and gas atmosphere.[92]

The use of combined inhibitors is important organoleptically. A food preserved solely by high salt or low pH may well not be palatable, but lower salt concentration combined with less acid may be acceptable. Similarly, mild heating of a food of low a_w may give a better textured product than the high heat required to stabilize a food of high a_w.

Much of the research on combination treatments has involved foodborne pathogenic bacteria and xerophilic fungi. Examples of pH and a_w interactions are provided by *Clostridium botulinum*. At 37°C, growth of type A occurred at a_w levels down to 0.94 at pH 7, but at pH 5.3 the lower limit was 0.99.[9] For proteolytic type B at 40°C, the lower limits were 0.94 at pH 7 and 0.96 at pH 6.[147] Two types of temperature effect are illustrated by an osmophilic yeast. Minimum a_w levels for fermentation were 0.66 at 30°C, 0.70 at 20°C, and 0.78 at 10°C.[229] In contrast, multiplication occurred at 40°C only if the a_w was substantially reduced by salt or sugar.[149]

Considerable attention has been paid to meat preservation systems, involving a_w or NaCl, pH, and sodium nitrite. This has stemmed largely from the trend to reduce the levels of salt and nitrite in the diet (see *Meat* under Section 7.9.2). The foodborne bacterial pathogens on which such studies have been reported include *Clostridium botulinum*,[135,175] *C. perfringens*,[75] *Escherichia coli*,[74,209] *Listeria monocytogenes*,[26,58,133] *Salmonella* spp.,[73,74] *Staphylococcus aureus*,[27,210] and *Yersinia enterocolitica*.[17,208]

Classic examples of combined preservation are the intermediate moisture foods (IMF). Definitions of IMF abound, an early description being foods of 0.7 to 0.9 a_w with water contents of 20%–50%.[104] Essentially, the a_w of the food is reduced to a level where pathogenic and most spoilage bacteria will not grow (this a_w may be increased, eg, by reducing pH), and fungi are controlled by chemical mycostats or some other inhibitory factor. The result is a food that is microbiologically stable but sufficiently moist to be consumed without rehydration.[219] As examples, meat products, including fermented sausages, may be preserved by low pH and low a_w alone.[121] Among intermediate moisture (IM) fruits, dried (but moist) apricots are preserved by reduced a_w and the inhibitory effect of residual sulfite applied to control browning.[219] Heat plays a vital role in the preservation of many foods in this category. Commonly this involves a mild heat treatment that inactivates vegetative cells, packaging that prevents recontamination, and levels of a_w, pH, Eh, chilling, preservatives, or other factors that prevent outgrowth of surviving spores.[219] Mathematical modeling of the combined effects of a_w, pH, and Eh on heat destruction of bacteria and yeasts has given good correlation with measured values.[166]

The concept of considering all practical preservation factors together, the level of inhibition or destruction caused by each, and the order in which they are applied, has been developed by Leistner as the "Hurdle Concept."[118,121] Factors most commonly considered in the hurdle concept, besides a_w, have been pH, temperature (high and low), Eh, oxygen tension, modified atmospheres, competitive flora, and a wide range of preservatives.[118] Other potentially useful treatments or components of such systems are pressure, ultrasound, radiation, and microstructure.[118]

Pioneering studies on IMF involved formulation of hennican, a chicken-based adaption of the buffalo meat and berry food prepared by American Indians.[1] The concept was also applied to the formulation of moist pet foods.[102] More recent applications of hurdle technology have involved meat products[117,118] and refinement of traditional Latin American IM foods.[3] In the former, shelf-stable (SS) meat products were classified as F-SSP, a_w-SSP, and pH-SSP, being foods stabilized predominantly by heating, reduced a_w, and reduced pH, respectively.[117]

An example of F-SSP is autoclaved sausage, where the heating (20 to 40 minutes at 103°–108°C) causes sublethal damage to surviving bacterial spores, the

a_w inhibits surviving spores, the Eh inhibits a_w-tolerant *Bacillus* species, and the pH is mildly inhibitory. Residual nitrite (after heating) may contribute to the inhibition. The a_w-SSP are exemplified by Mortadella, where reduced a_w is achieved largely by formulation, and Bruhdauerwurst, where it is achieved largely by drying.[117] With heat treatments to center temperatures of 75°–78°C, surviving bacterial spores are inhibited by an a_w of 0.95.[117] Mold growth on the casings may be controlled by smoking or, more efficiently, by application of sorbic acid.[117] The pH-SSP include brawns, which are acidified to pH 4.5–5.0 with acetic acid, and a Bologna-type sausage acidified to pH 5.4–5.6 with gluconic acid derived by hydrolysis from glucono-delta-lactone added to the formulation. For stability without refrigeration, the latter is reheated and vacuum packed at 80°C for about 1 hour.[117] Clearly, there is considerable overlap between these processes, most of them being dependent to some degree on combinations of heating, reduced a_w, and reduced pH.

In the study of traditional Latin American IM foods, analyses of individual foods and comparisons among similar foods from different regions enabled improvements to be made in stability and organoleptic quality, largely by increasing the range of hurdles employed and by introducing mild heating procedures in many cases.[3]

7.8 SPECIFIC SOLUTE EFFECTS

As noted earlier, the application of the a_w concept to microbial growth in concentrated environments rests on the assumption that the effects observed are solely a function of a_w.[41] However, in some instances, responses at a given a_w differ when different solutes are used to control the a_w value. These have been termed *specific solute effects.*[41] The tolerance of specific solutes by halophilic bacteria and some xerophilic fungi have already been referred to and, in the case of halophiles, explained.[21] Much less dramatic effects can also be of concern. When *Salmonella oranienburg* was grown in a glucose-salts medium, additions of NaCl, sucrose, and a mineral salts mixture produced comparable growth inhibition at all a_w levels tested.[40] Glycerol, however, was much less inhibitory on an a_w basis. Similar responses have been recorded for many bacteria, including other Gram-negative rods, aerobic, and anaerobic spore formers and *Listeria* (Table 7-5).

Such data indicate[69,78] that, in concentrated environments, microbial growth is subjected to solute interactions in addition to a_w, and suggest that other parameters may better describe the relationship between microbial activity and these environments. This picture is complicated by the fact that there are other bacteria, for example, some Gram-positive cocci, to which glycerol is more inhibitory than NaCl on an a_w basis,[130] a fact that has been largely ignored in the discussion of specific solute effects. The most important of these glycerol-sensitive, Gram-positive cocci in foods is *Staphylococcus aureus*. The influence of a range of other organic solutes and of NaCl on *S. aureus* has been studied in some detail. Three facts emerged: (1) as with glycerol, growth was inhibited at higher a_w in ethanol, 1-3 butylene glycol, propylene glycol, and several polyethylene glycols than in NaCl or sucrose,[222,223] (2) cells died rapidly in all of these solutes except NaCl and sucrose,[12] and (3) ethanol, the diols, and the polyethylene glycols adversely affected the integrity of the bacterial cell wall.[12] There was no correlation between the effects of solutions of sucrose, glucose, NaCl, glycerol, or ethanol on *S. aureus* and their influence on viscosity, dielectric constant, or oxygen solubility or diffusivity in solution at the relevant a_w levels.[12] The streptococci did not appear to share with *S. aureus* this sensitivity to glycerol.[182]

Most of the data on specific solute effects relate to differences in minimum a_w levels permitting growth. Equally or more striking differences may be observed in growth rates in the various solutes at a_w levels near the mid-range for growth. *Eurotium amstelodami* grew optimally at 0.96 a_w in several solutes, but the rates of growth were almost twice as high when a_w was controlled by sucrose or glucose as when it was controlled with NaCl or glycerol.[186] *Salmonella oranienburg* had a similar minimum a_w for growth (0.95) whether salts or sucrose were the bulk solutes, but at 0.97, growth was twice as rapid in salts as in sucrose.[44] Studies of nutritional requirements at this a_w suggested that certain nutrients that stimulated growth rate in salt were not utilized in sucrose.[39,41] Major differences in growth rate at such intermediate a_w levels were also observed with *Bacillus subtilis* and *B. stearothermophilus*.[41] These appear to be profitable regions in which to explore specific solute effects.

There is not yet a parameter that will correlate better than a_w with the effects of concentrated solutions on microbial growth. It is difficult to suggest where to seek such a parameter in view of the fact that some bacteria appear, in terms of a_w, to be excessively resistant to glycerol while some are excessively sensitive, if bacterial reactions to NaCl are considered "normal." The data of Marshall et al.[130]

Table 7-5 Influence of Solute on the Minimum Water Activity Permitting Bacterial Growth

Bacteria	*Solute*	*Minimum* a_w	*Reference*
Bacillus cereus	NaCl	0.93	130
	Glycerol	0.92	
Bacillus megaterium	NaCl	0.94	130
	Glycerol	0.92	
Clostridium botulinum type A	NaCl	0.94	9
	Glycerol	0.92	
Escherichia coli	NaCl	0.95	130
	Glycerol	0.94	
Lactobacillus helveticus	NaCl	0.95	182
	Glycerol	0.93	
Listeria monocytogenes	NaCl	0.92	211
	Glycerol	0.90	
Salmonella oranienburg	NaCl	0.95	130
	Glycerol	0.94	
Streptococcus lactis	NaCl	0.97	182
	Glycerol	0.92	
Bacillus subtilis	NaCl	0.90	130
	Glycerol	0.93	
Micrococcus species MR1	NaCl	0.83	130
	Glycerol	0.92	
Staphylococcus albus	NaCl	0.88	130
	Glycerol	0.90	
Staphylococcus aureus	NaCl	0.86	130
	Na acetate	0.86	223
	Sucrose	0.87	41
	Glycerol	0.89	130
	Xylitol	0.93	223
	Erythritol	0.95	223

suggest that two bacteria could exist with identical growth responses to NaCl, but with one being more sensitive to glycerol than to NaCl on an a_w basis and the other less. In fact, *Micrococcus lysodeikticus* and *Listeria monocytogenes* both grow to 0.92 a_w in NaCl medium, but in glycerol-adjusted medium the former grows to 0.93 a_w[130] and the latter to 0.90 a_w.[211] This appears to be a "specific cell response" rather than a "specific solute effect," and one that would presumably be difficult to predict from a knowledge of solution properties. For the present, it seems logical to accept that for practical purposes the effects on microorganisms of those solutes most likely to be added to foods in high concentrations, NaCl and sucrose, are adequately described in terms of a_w.

7.9 PRESERVATION OF FOODS AT REDUCED a_w

Control of a_w, either alone or in combination with other inhibitory factors, is the means of preservation of a wide range of foods against both microbial food spoilage and the growth of pathogenic and toxigenic microorganisms. In some cases, the growth of organisms, selected partly or wholly by reduced a_w, plays a major role in food preservation. When such foods are inadequately processed or stored, spoilage follows a pattern determined largely by the prevailing a_w. The following sections consider only the a_w-dependent microbiological aspects of processes. For detailed descriptions of the other microbiological involvement in food processing, see the relevant commodity chapters.

7.9.1 Plant Foods

Cereals, Nuts, and Legumes

The seeds of all cereal, nut, and legume crops lose moisture during maturation. Most are harvested at a_w levels below those supporting microbial growth. However, some, particularly grains, may be invaded by field fungi on the plant prior to maturity when the a_w is still high ($\geq$ 0.95).[37] Such invasion may result from damage by insect larvae, as in the case of *Aspergillus flavus* infection of corn kernels.[38] As grains mature, fungal growth ceases and slow death follows. However, if the grain remains moist, growth continues and some field fungi may produce mycotoxins.[30] The most xerophilic of the mycotoxigenic molds appears to be *Aspergillus ochraceus*. Its minimum a_w for growth is below 0.76, and for the production of the toxins penicillic acid and ochratoxin is 0.80 and 0.85, respectively.[6] Few of the strains that frequently contaminate grains are toxigenic.

If drying of grains is not sufficiently rapid, or if grains are remoistened, they may be attacked by storage fungi. These are capable of invading kernels at minimum levels of a_w that range from 0.85 for some species to less than 0.70 for others[219] (see Section 7.4.2). These storage fungi are by definition[154] xerophiles. They include species of *Aspergillus, Eurotium, Penicillium, Wallemia,* and *Chrysosporium*[88] (Table 7-4).

The recommendation of water contents for commodities as "alarm," "safe," or "limiting" to ensure that fungal spoilage will not occur can be misleading.[186] The slope of many water sorption isotherms is relatively flat over the range 0.6–0.8 a_w, and in some commodities, a change in water content of only 1% can result in an a_w change of 0.04–0.08. This uncertainty can be compounded if the form of the commodity is not given, as in the case of peanuts, which may be traded as kernels or in shell.[106] Here the water contents of kernels and of peanuts in shell at 0.75 a_w were about 9% and 12%, respectively. Some of these crops, eg, peanuts, are harvested while still moist, with a_w levels above 0.90. Mold attack is very likely unless the crop is dried rapidly to a safe a_w. Of particular concern are the aflatoxigenic species, *Aspergillus flavus* and *A. parasiticus*, which grow at a_w levels down to 0.78. However, aflatoxin was not produced by *A. flavus* in inoculated, mature kernels incubated at 83% RH for 84 days.[55]

The problem of moisture migration in stored grain has already been referred to. Temperature gradients, eg, across silos, result in moisture migration as vapor from warm to cooler regions, resulting in a_w gradients that may permit mold growth in grain even if the average moisture content corresponds to a safe a_w.[232] The a_w of many cereal products, such as crackers, biscuits, and dry pasta, is too low to support microbial growth (Table 7-2). However, *Staphylococcus aureus* can multiply in the early stages of pasta production and can reach hazardous levels if drying is slow.[224]

Vegetables

Many fresh vegetables have high a_w and will spoil readily during extended storage unless refrigerated. For some, especially leafy, green vegetables, a_w cannot be reduced sufficiently to greatly restrict microbial growth without wilting and loss of quality. When vegetables are dried to produce a stable product, the a_w required to minimize organoleptic changes is far below that necessary to inhibit all microbial growth. These a_w levels are in the range 0.35–0.10. Most vegetables enter the drying plant carrying heavy microbial loads, mostly of soil origin. Treatments preparatory to drying will destroy the vast majority of these, ie, the lye treatment of tomatoes and some root vegetables and the blanching or precooking of others.[219] Onions, which are not blanched, can enter the dehydrator heavily contaminated.[227] Potatoes, onions, and peas are the most commonly dehydrated vegetables.

Many vegetable foods are preserved by pickling, a process in which microorganisms growing in the food produce sufficient acid to inhibit the growth of spoilage and pathogenic agents. NaCl is added to select these desirable lactic acid bacteria. It does not specifically inhibit those microorganisms causing spoilage or foodborne disease, but is generally more inhibitory to them than to the lactic acid producers, *Leuconostoc* spp. being the most sensitive of these, with lactobacilli and pediococci showing greater resistance.[146] Single or mixed cultures of lactic acid bacteria and sometimes yeasts may be added as starter cultures. During fermentation, the fermentable carbohydrate is depleted, which limits the growth of acid- and salt-tolerant bacteria and yeasts, while the low pH controls salt-tolerant micrococci.[70]

Common fermentations in which NaCl, and hence reduced a_w, is used as a selective agent are applied to cabbage (to produce sauerkraut), to cucumbers, to olives, and to soybeans (to produce soy sauce) (see Chapter 26). To prepare sauerkraut, shredded cabbage is brined under anaerobic conditions with 2% NaCl, an a_w of 0.988. This low concentration permits growth of *Leuconostoc mesenteroides*, the least salt tolerant of the lactic acid bacteria involved. It is succeeded by *Lactobacillus plantarum* and *L. brevis*.[152] Low temperature fermentation (13°–18°C) permits use of lower salt concentrations (0.987–0.990 a_w), which favor growth of heterofermentative species (*L. brevis, L. mesenteroides*) while also permitting homofermentative activity (*L. plantarum, Pediococcus cerevisiae*). This is said to give the highest quality product.

Cucumbers are pickled in higher initial salt concentrations (5% to 8%; 0.97–0.95 a_w) to inhibit *Leuconostoc mesenteroides*. After one week, the fermentation is complete and salt is added progressively to give a holding brine of about 17% NaCl (0.87 a_w).[60] Olives are first lye treated to hydrolyze the bitter glucoside, oleuropein. Otherwise, the process is similar to the pickling of cucumbers, but with *L. mesenteroides* playing a more important role. In both cucumber and olive fermentations, a potential problem is the growth on the surface of the brine of oxidative film yeasts. These may oxidize lactic acid with increase in pH, permitting growth of coliform bacteria.[60] These yeasts, which include *Debaryomyces* spp., are very tolerant of reduced a_w, and control depends on exclusion of oxygen from the fermentation.

Reduced a_w is important in the second stage of soy sauce production.[149] In the first stage, soy beans or soy beans with wheat flour are inoculated with *Aspergillus oryzae* or *A. soyae*. After about three days incubation at ambient temperature, the mat of molded beans is submerged in a strong brine (18% NaCl; 0.86 a_w). Here, the fungal enzymes continue to act on the mash and salt-tolerant bacteria grow and reduce the pH from 6.0 to below 5.0. The xerophilic yeast, *Zygosaccharomyces rouxii*, multiplies and contributes to the final flavor of the sauce. The whole process takes in excess of one year.[219]

Some other vegetables (eg, green tomatoes, green beans, and corn) may also be preserved by fermentation. However, vegetables that contain insufficient fermentable carbohydrate to support a strong lactic fermentation, and those that soften readily, are more commonly salted in very concentrated brines (0.86–0.75 a_w), which prevent the fermentation.[219]

Fruit

Most fresh fruits are in the acid range (pH 4.5–3.7) and are susceptible to spoilage by lactic acid bacteria, yeasts, and molds. Reduction of a_w by drying or syruping controls the bacteria, but many species of fungi are both xerotolerant and acid tolerant. Hence, fruit preserved solely by drying must have an a_w of 0.65 or below. Sulfur dioxide, in the form of sulfites, is added at high concentrations to control nonenzymic browning in some light-colored fruits.[172] The residual levels of sulfite of 1,000–3,000 ppm will prevent fungal spoilage even at relatively high a_w.[219] The use of preservatives such as sorbic acid also assists in providing microbial stability.

If dried fruits, such as prunes, rehydrate to a_w levels above ~0.68 spoilage may ensue, the most likely agents being xerotolerant *Eurotium* spp. High moisture prunes of 0.94 a_w, intended for consumption without rehydration, are prone to spoilage by *Eurotium* species and by *Xeromyces bisporus*, unless treated with an appropriate antimycotic or heated in a hermetically sealed container.[155] *X. bisporus* may grow at a_w close to 0.60.

Fruit products, such as jams and jellies, are heated for long periods during preparation. This not only destroys vegetative microorganisms, but also lowers a_w by partial inversion of the sucrose present. A final a_w of 0.80 or below is sufficient to prevent growth of surviving organisms, although surface growth of fungi may result from recontamination.[219] Liquid fruit products, such as fruit and juice concentrates, syrups, and cordials, although of low a_w and low pH, are difficult to preserve without heating, chilling, freezing, or the use of chemical preservatives. A pear concentrate, without preservative but of pH 4.5 and 0.62 a_w, supported growth of the xerotolerant yeast *Zygosaccharomyces rouxii*.[229] Both sorbic and benzoic acids should control *Z. rouxii* in acid products at any a_w. However, *Zygosaccharomyces bailii*, a very preservative-resistant yeast, is a common spoilage agent of such products at a_w above 0.80.[161]

Reduced a_w may also play an important role in the microbial stability of canned fruit. These receive a mild heat treatment, primarily because the low pH restricts outgrowth of surviving bacterial spores. However, the reduction in a_w provided by the canning syrup probably makes a synergistic contribution with pH. Growth of the butyric anaerobe *Clostridium pasteurianum* occurred in canned pears in syrup at pH 3.8 if a_w was ≥ 0.985, but not at pH 3.8–4.0 when a_w was 0.985–0.975.[95]

Sugar, Syrups, and Confectionery

Sugar, widely used in food preservation to reduce a_w, does not undergo microbial spoilage in its dry, crystalline state. Storage at high humidity results in surface hydration and formation of a saturated sucrose solution of 0.86 a_w. This a_w is within the growth range of xerophilic fungi, but in the absence of adequate noncarbohydrate nutrients spoilage does not occur. The microorganisms of most importance in sugar are the thermophilic bacterial spores that, when contaminated sugar is used in the formulation of canned foods, can survive the heat process and cause post-processing spoilage.[141]

Liquid sugar, which is either sugar concentrated after the decolorization step in refining or is crystalline refined sugar dissolved in water, has a sugar content of 66°–76° Brix, depending on the extent of inversion.[93] At 66° Brix, the a_w is that of a saturated sucrose solution (0.86), and the syrup can support slow growth of xerophilic yeasts, particularly if a_w gradients occur during storage. "Totally" inverted syrups (72% to 73% solids of which 5% is sucrose) have much lower levels of a_w and much greater stability. The syrups derived from the hydrolyzed starch of corn or other plants have colligative properties similar to sucrose invert sugars of similar concentration. Honey is harvested from the comb at about 0.60 a_w. The main cause of spoilage after some rehydration has been *Zygosaccharomyces rouxii*.[231] The heat treatment that is now given to honey to prevent glucose crystallization effectively sterilizes it.[159]

Confectionery, which includes chocolates, toffees, and candies, is normally resistant to microbial attack as a consequence of a_w levels mostly in the range of 0.65–0.50. Although the low a_w of chocolate precludes microbial growth, it and the high fat content protect vegetative bacteria from destruction by heat during the conching process. Salmonellae surviving in chocolate have been the cause of several disease outbreaks[76] (see Chapters 35 and 45). In some confectionery, the a_w is much higher.[93] Thus, fondants may be in the a_w range 0.84–0.75. When used in soft-centered chocolates, gas-producing *Clostridium* spp. have caused the product to explode.[235] Inverting the fondant with invertase will lower the a_w sufficiently to control these bacteria, but *Zygosaccharomyces rouxii* can also cause this problem and is much less readily inhibited by reducing a_w. Control of this organism demands thorough cleaning of the filling lines.[159]

7.9.2 Animal Foods

Fish

Fresh fish is a highly perishable commodity and a variety of traditional procedures that involve a_w reduction have been developed for preservation (see Chapter 21). These include salting, drying, and smoking. In salting, suitably prepared fish are stacked in layers interspersed with salt. (If the fish has not been gutted, autolysis occurs, a process utilized in the production of fish sauces.) Salt penetrates the tissues and water is withdrawn, diluting the brine. This brine may be drained to give a dry salted product, or permitted to build up until the fish is covered. Lost salt is replaced to maintain near saturation of the brine.[219] The salted fish may be dried and/or smoked.

Heavily salted fish are susceptible to spoilage by the halophilic archaebacteria *Halobacterium* and *Halococcus*, capable of growth in a saturated NaCl environment. Spoilage is proteolytic with pink discoloration. These bacteria are aerobic and unable to multiply below 10°C, so exclusion of air or refrigeration provides control. The most common fungal spoilage agent of salt fish in temperate regions appears to be *Wallemia sebi*, the "dun" mold,[68] which can be controlled by dipping fish in a 1% solution of sorbic acid.[219] In tropical regions, *Polypaecilum pisce* predominates.[158,237]

Fish may be hot (65°–90°C) or cold (20°–45°C) smoked after salting. Smoke constituents aid in the preservation of fish, but most of the preservative action comes from the salting and drying treatments. Very lightly smoked white fish was responsible for an outbreak of type E botulism in the United States (US), although undercooking and poor sanitation were probably more important factors.[219] *Clostridium botulinum* type E did not grow below 0.97 a_w.[9,147] Thus, fish salted to less than 5% will require refrigeration to ensure safety from this pathogen. It multiplied at temperatures close to 3°C in the absence of salt,[184] but at 0.97 a_w growth was prevented at 15°C.[148]

Gutted fish may be salted and held in saturated brine for long periods. Exclusion of air prevents growth of red halophilic bacteria, although slow autolytic changes occur.[219]

Meat

Fresh raw meat has an a_w in excess of 0.99 and is highly perishable unless chilled or frozen.[188] Water activity plays a role in the preservation of chilled meat, as the drying that accompanies initial chilling lowers the surface a_w rapidly. At an air temperature of 2°C under normal abattoir conditions of airspeed, humidity, and initial carcass temperature, the a_w of the lean meat surface fell to 0.76 in 30 minutes and took four hours to rehydrate to 0.95 a_w.[123] As the main spoilage microorganisms of chilled meat are Gram-negative psychrotrophic bacteria such as *Pseudomonas* species with lower limits for growth as high as ~0.97 a_w,[130] substantial inhibition results.

Dried meats (eg, charqui, carne secca, and biltong) are prepared traditionally in many countries by sun drying. The major requirement is that the meat is in well separated thin pieces so that the interior, as well as the surface, dries rapidly enough to limit bacterial growth. *Eurotium* spp. were dominant on South African biltong, followed by *Aspergillus* and *Penicillium* spp.[226] More modern technology provides two common drying techniques. In the first, cooked minced meat is dried in hot air tunnels at temperatures exceeding 50°C.[190] Again, speed of drying is important to prevent bacterial growth in cooler, wetter regions. Alternatively, fresh or cooked meat may be freeze dried, or dried from the frozen state. As microbial growth is not a problem in frozen meat, larger pieces can be dried by this process. Little change in microbial load occurs.[93]

Cured and Fermented Meats

There is a wide variety of cured meats and meat products, especially pork and beef. Modern meat curing practices stem from traditional preservation of red meats with salt contaminated with sodium nitrate (see Chapter 19). While salt was the principal preserving agent, salt-resistant bacteria, chiefly micrococci, reduced nitrate to nitrite that reacted with meat pigments, mainly myoglobin, to yield the insoluble red pigment nitric oxide myoglobin.[92] Heating converted this to the pink nitrosyl haemochrome. Modern curing technologies do not rely on the reduction of nitrate by bacteria but add nitrite with much purer NaCl. In general, about 100–200 ppm sodium nitrite is added to cured products, although in some countries much lower concentrations are permitted.[93]

Cured meats are of three broad types: (1) unheated, cured meats, which generally do not require refrigeration, except for some lightly cured hams, sausages, and bacon, (2) products mildly heated to a center temperature of 65°–75°C, which require refrigeration after heating, and (3) severely heated products, which are shelf stable after heating to 100°–120°C.[92] (See also IMF discussion in Section 7.7.) Curing agents do not have a marked effect on the destruction of microorganisms. However, in unheated products they inhibit the growth of undesirable organisms such as salmonellae. In pasteurized products they help to control growth of heat-resistant vegetative bacteria and, in severely heated cured meats, they inhibit the outgrowth of surviving bacterial spores.[92]

The bacteriostatic effect of nitrite in cured meats is greatly affected by pH. It increases as pH decreases from 7.0 to 5.0 and equates well with the concentration of undissociated nitrous acid.[31] In practice, there is a 5- to 10-fold increase in bacterial inhibition per pH unit as pH is reduced over this range.[174]

The microbiology of cured meats is conveniently considered in terms of high and low a_w products, the dividing a_w being 0.92, equivalent to ~10% salt in water.[93] High a_w cured meats include bacon and raw ham and semi-dry fermented and unfermented sausages. Because of relatively low salt content, all require refrigeration for useful shelf life. Even in these lightly cured products of ~ 0.97 a_w, the Gram-negative bacterial rods that predominate in the microflora of raw meats are inhibited. Spoilage is mainly by lactic acid bacteria, which produce souring and green discoloration.

Sliced, high a_w cured meats, such as ham, are readily contaminated in handling and from slicing machines by *Staphylococcus aureus*, which, being salt tolerant, is favored by reduced a_w. Such ham has been a frequent vector for staphylococcal enterotoxin.[87] Smoking of frankfurters, also of high a_w, may leave micrococci predominant, which will multiply in refrigerated storage.[219]

The emergence of *Listeria monocytogenes* as a foodborne pathogen has focused attention on the presence of this psychrotroph in chilled meats. The study of interactions between pH, a_w, nitrite, and ascorbate on growth rate and lag time of *L. monocytogenes* on vacuum packed, sliced, cooked meats makes possible the prediction of safe shelf lives for these products.[58]

High a_w fermented sausages depend for microbiological stability mainly on the acid produced by lactic acid bacteria and the consequent lowering of pH. If produced without added starter culture, the dominant fermenters are usually lactobacilli such as *Lactobacillus plantarum*.[54] However, pediococci such as *Pediococcus cerevisiae* are the preferred starters for some semi-dry sausages.[53] The ultimate pH of fermented sausage is usually in the range 4.5–5.0.[197]

Low a_w fermented sausages, such as many salamis, are generally not smoked or cooked and are eaten raw. They are subjected to longer drying periods than high a_w sausages. *Lactobacillus plantarum* is again usually the dominant bacterium if starter cultures are not added. *Pediococcus* spp. are desirable starters.[153] However, in both German and Italian salami-type sausages, even if used as a starter, *L. plantarum* did not survive in the fermentation. *Staphylococcus carnosus*, used in both countries as a starter, grew well in the products, which had final a_w values of 0.92/0.93 (German) and 0.88/0.91 (Italian).[128] Dry sausages are frequently coated with molds such as *Penicillium*, *Scopulariopsis*, and *Aspergillus* spp.[119]

Large cuts of meat, such as beef silversides and pork legs and sides, are pumped with curing solutions before being submerged in brine tanks to complete the curing process for corned beef, ham, and bacon, respectively. The curing period for corned beef is much shorter than for pig meats. The brines generally contain over 15% NaCl, being much higher for bacon than for ham.[219] Several moderately halophilic bacteria, including *Micrococcus halodenitrificans* and *Vibrio costicola*, both with lower limits of ~0.86 a_w, can grow in slightly less concentrated brines and cause spoilage.[13]

A detailed description of the manufacture of prosciutto (raw ham) followed the chemical, physical, and microbiological changes during curing.[10] The success of the process, in terms of microbiological safety, could only be explained if the various physicochemical properties were taken to act additively or synergistically, since none, individually, would inhibit pathogenic bacteria with certainty. In ham that was cured but not dried (0.95 a_w), staphylococcal enterotoxin was produced in inoculated samples at ambient temperatures within 7 days.[221] When dry cured to 0.92 a_w, toxin was produced within this period only at temperatures above 20°C. If dry cured to 0.89 a_w, enterotoxin production occurred only at 37°C within seven days.

Concern over decreases in the concentrations of salt and nitrite in the curing of meats has lead to extensive studies on the fate of *Clostridium botulinum* in such products. As with raw hams, the interactions between preservative factors are complex (see Section 7.7). In the control of *C. botulinum*, there is a reciprocal relationship between salt and nitrite. In absence of nitrite, 7% salt was required to prevent growth of vegetative cells of type A (5×10^3 cells ml^{-1}) for three months at 35°C; while with 300 ppm nitrite, total inhibition was obtained with 3% salt.[73,175] Reduced pH will also contribute to the inhibitory action, but, as with NaCl as an agent of a_w reduction, there are organoleptic limits of acceptability, which differ between foods. It has been pointed out[131] that many of the studies of factors in combination (see Section 7.7) are relevant only to the product or conditions tested. A better approach is the development of a database of responses of microorganisms to environmental factors encountered in foods, so that predictions of response under untested or new conditions may be made. This approach, termed *predictive modeling,* is discussed in several sources[11,131,180] (see also Chapter 16).

Dairy Products

The main dairy products preserved wholly or partly by reduced a_w are whole and nonfat dried milk, condensed milk, and a wide variety of cheeses. Spray drying is now the most common milk drying process. The product is about 0.2 a_w, too low to support any microbial growth, but the reconstituted product is as perishable as raw milk. After pasteurization to destroy vegetative bacterial pathogens in the raw milk, it is concentrated to 40% to 50% solids and held in a balance tank. If held there too long at temperatures below 65°C, growth of *Staphylococcus aureus* may occur, favored by the reduced a_w, with the possibility of enterotoxin production. The spraying process dispenses milk droplets into a current of dry air at 150°–260°C.[93] In spite of the high air temperature, some survival of vegetative as well as sporing bacteria occurs.[215] The drying process is thus not a substitute for adequate pasteurization and sanitation. Salmonellae have been a particular problem in dried milks and in related infant foods. They have been introduced into dried milks both through cracks in the metal drying cone and, more commonly, from the environment after drying and during packaging.

Sweetened condensed milk contains concentrated milk and sucrose with an a_w of 0.85–0.89. Mild heating during concentrating effectively pasteurizes the product, but no heating occurs after canning. Fungal contaminants may cause gas production from growth of a xerophilic yeast, *Torulopsis lactiscondensi*[219] or mold colonies (buttons) from growth of *Aspergillus* or *Penicillium* spp. if there is adequate air in the head space.[93]

Cheese is a concentrated form of fermented milk obtained by the coagulation of casein.[93] The many types may be classified as unripened soft cheeses and ripened hard-grating, hard, semi-soft, and soft cheeses; processed cheese and cheese spreads; and whey cheeses.[67] These cheeses vary greatly in moisture content and hence in a_w.

The microbiology of all cheeses begins with the starter culture, which may be any of a range of *Lactobacillus* and *Lactococcus* spp., sometimes also *Propionibacterium* or *Leuconostoc* spp.[93] The major microbiological problem during fermentation is attack by bacteriophage on the starter bacteria, which diminishes lactic acid production. The expulsion of water after coagulation and cutting of the curd reduces water content, but the expelled water carries with it much of the salts and lactose that would reduce a_w.[219] Reduction of a_w is achieved by addition to the curd of 1.5% to 5% NaCl, which also enhances flavor.

The most comprehensive report on the a_w of cheeses in relation to composition and microbiology is from Rüegg and Blanc.[182] Analysis of 19 cheese types gave typical a_w levels ranging from 0.988 (cottage cheese) to 0.917 (Parmesan). The a_w levels in hard cheeses ranged from 0.948–0.972, for semi-soft cheeses from 0.95–0.977, and for soft cheeses about 0.98. The authors point out the changes in a_w that continue during ripening of hard cheeses and the pronounced internal a_w gradients that can still exist after six months aging. The a_w of rinds could be very much lower than in the interior. A later report from the same source[181] included much lower a_w values as typical of hard cheeses, 0.86–0.97. The latter a_w values were in accord with those determined in samples obtained from US supermarkets[219] and for those reported for hard Spanish goats' cheese after 16 weeks ripening, where the range of a_w values was 0.88–0.93.[216] The moisture contents in these cheeses were described as very low, because of low relative humidity in the ripening rooms. The micrococci that grow on the surface of Roquefort cheese, which are partly responsible for the specific aroma of this ewes' milk blue vein cheese, multiplied in 20% NaCl (0.88 a_w), and are clearly well adapted to this environment.[228]

7.10 DETERMINATION OF WATER ACTIVITY

A knowledge of the a_w of a food can be vital in determining its microbiological stability and safety. Raoult's Law, describing the relationship between solute concentration and a_w (see Section 7.2), holds only for ideal solutions and then only at concentrations below 1 molal. For nonideal solutes, such as electrolytes generating two or more ions per molecule, a_w may be calculated using the equation

$$\log_e a_w = -vm\phi/55.51 \qquad (5)$$

where v is the number of ions generated per molecule of solute (and is 1 in the case of nonelectrolytes), m is the molal concentration, and ϕ is the molal osmotic coefficient for which values are recorded in the literature.[219] Many models have been devised for the prediction of the a_w of nonideal solutions, and 11 have been reviewed.[164] The models most used in relation to foodstuffs appear to be those of Roos,[179] and the development from this of Caurie,[32] which can handle solutions with mixed solutes; of Norrish[144] for the nonelectrolyte solutions; and of Grover[82] developed from studies of confectionery solids.

Foods are often very complex mixtures that do not lend themselves to the application of such models. However, foods may be formulated to a desired a_w from a knowledge of their water sorption isotherms (see Figure 7-2), and the production of such isotherms demands measurement of a_w. Many methods of measurement exist, most based on the determination, directly or indirectly, of the relative humidity of the atmosphere in equilibrium with the food sample. The methods used for measurement should be both accurate and reproducible. Other desirable attributes are speed, cost, ease of use, durability, and, in some cases, portability.[219] The most inconvenient factor in measuring a_w by equilibration methods is the often inordinately long time taken to reach equilibrium. This is minimized by keeping the volume of the equilibrating atmosphere, or head space, as small as practicable. Detailed data are available on the a_w of selected saturated salt solutions at 25°C for use in equilibration or calibration,[169] on the equilibrium relative humidity values of selected saturated salt solutions at a range of temperatures,[81] on the a_w of unsaturated solutions of salts and of sulfuric acid solutions,[36,168] and on other properties of water.[164]

The methods of a_w measurement in regular use in food research laboratories are as follows[189]: vapor pressure manometers, simple gravimetric and hygrometric methods, dew point, freezing point, fiber hygrometers, and electric hygrometers.

Extensive reviews of these methods were done by Kress-Rogers[109] and Troller.[218] The report of the most comprehensive interlaboratory testing of methods widely used at the time[205] should be consulted for details on the accuracy and precision of those methods. Other comparisons were done by Fernandez-Salguero et al.[64] Performance criteria for electric hygrometers and dew point instruments were done by Rahman[164] and Troller and Scott.[220] There is a need for instruments to measure a_w in foods at higher temperatures and pressures.[164]

7.10.1 Vapor Pressure Manometers (VPM)

The vapor pressure and hence a_w of a food may be measured by manometric procedures with a precision of ±0.005 a_w below 0.85 a_w, but above this a_w accuracy is lower (±0.02) because of temperature control difficulties.[230] Problems may also be caused at high a_w by condensation.[230] A ground food sample is maintained in the sample chamber at ~ −80°C, while the attached manometer is evacuated. The sample is warmed while one side of the manometer is maintained at near zero pressure. The increase in pressure caused by the vapor pressure of the sample alters the fluid level in the sample arm and the reading taken.[219] Vapor pressure measurements should be sensitive to 0.01 mm Hg.

Advantages of the manometric method include the ability to conveniently carry out multiple vapor pressure determinations on the same sample,[213] which simplifies the construction of water sorption isotherms. The manometers are relatively inexpensive, but can be cumbersome and fragile. The method is inappropriate for use with materials that are respiring or fermenting or that expand excessively under high vacuum. Factors controlling the accuracy of the VPM method are discussed by Lewicki et al.[122] They require absolutely uniform temperatures and a high degree of thermometric accuracy.[219] However, tests involving three laboratories in which results obtained with seven methods (direct measurement by VPM, an isopiestic technique, and several electronic devices) were compared indicated that the VPM method was probably the best and most accurate.[112] Tests with three commercial electronic instruments gave an average of 0.051 a_w units difference from VPM values.[202] It was concluded from these studies that a_w values quoted to three decimal places are of questionable validity. The VPM method is frequently used as the standard with which other methods and devices are compared. However, data from an interlaboratory test of a number of a_w measuring instruments and methods revealed the effects of poor temperature control and insensitivity of the pressure measurement in the use of this method.[205]

7.10.2 Simple Gravimetric and Hygroscopic Methods

A simple method[114] requiring little equipment involves determining by interpolation the a_w at which a sample would not lose or gain water. Food samples, usually four, are weighed and placed in separate relative humidity chambers, each containing a solution of different a_w. After one to two hours, water gain or loss is determined gravimetrically and the change plotted against a_w. The plot intersects the line representing zero moisture change at the a_w of the food. Ideally, two standards will have a_w on each side of the a_w of the sample. Saturated salt solutions are suitable as standards for ranging experiments, but, for the small incremental differences required for more accurate estimations, unsaturated solutions, eg, of sodium chloride,[36] may be more useful. A modified graphical interpolation method has been described.[98]

Isopiestic equilibration[176] is similar to the preceding method but the transfer of water vapor proceeds to constant weight, which may take one or more days. Good temperature control is essential and to minimize temperature differences between samples and standard solutions, these are placed in silver dishes that fit neatly into a copper block. An evacuated desiccator containing the copper block is rocked or rotated in a thermostated waterbath while equilibration proceeds. The water contents of sample and reference solution (usually of a salt) are determined, and the a_w of the equilibrated system is found from standard tables.[177] This is the a_w of the sample at its final water content, not in its initial state of hydration. The method provides accurate data for the construction of water sorption isotherms.

Equilibration of food samples with hygroscopic reference material for which a standard isotherm has been prepared also provides good estimates of a_w, particularly in the high a_w range, above ~0.90. Certain proteins[66] and microcrystalline cellulose[230] have been used as standards. The latter gave results comparable to those obtained with electric hygrometers and better than those from the manometric technique at high a_w.[230]

A modified application of this principle involves equilibrating the sample with about 0.01 g of a hygroscopic liquid (a polyol), measuring the refractive index of the liquid by refractometer, and determining the a_w from an ERH versus RI chart. The polyols used included glycerol, ethylene glycol, and propylene glycol. The method is simple and inexpensive. Readings are very rapid, but equilibration will be slow if the a_w of the polyol is far removed from that of the sample. Interference from volatile contaminants is negligible, and there is no hysteresis. The method lacks sensitivity below 0.30 a_w and equilibration time is excessive above 0.95 a_w.[203] No interlaboratory comparison of this method with others has been reported.

7.10.3 Dew Point Methods

Air may be cooled without change in water content until saturation is reached, when condensation occurs. The temperature at which this occurs is the dew point and it may be determined by observing or detecting condensation on a smooth, cooled surface. The dew point temperature is related to ERH or a_w by reference to psychrometric charts. Using thermoelectric cooling and photodetection cells, accuracies of ± 0.005 a_w are claimed. The determination is rapid,[170,171] but measurements can be affected by an unclean mirror surface and by condensibles that have lower critical temperatures than water. In a_w measurements by four methods on Brie and Camembert cheeses, a dew point hygrometer gave values lower by 0.003–0.004 a_w than either psychrometric or cryoscopic methods and showed greater differences from overall average values.[129] Note, however, that a_w values quoted to three decimal places are considered of questionable validity unless obtained by direct vapor pressure measurements.[112,202]

7.10.4 Freezing Point Depression Methods

The a_w of a solution may be determined from the depression of the freezing point relative to that of pure water. The vapor pressure of ice at various temperatures can be related to the vapor pressure of supercooled water.[219] The method is inexpensive, involving an ice-salt slurry of appropriate temperature and a thermometer with which to monitor the temperature change in the sample solution, held in a glass tube in the slurry. Supercooling of the solution usually precedes ice formation and a rise in temperature to the true freezing point. The method is most suitable for use with solutions,[65] although aqueous extracts and homogenates of solid foods, such as meat, have been used. There are difficulties in predicting a_w at temperatures in the microbiological growth range from data obtained near 0°C.[219]

7.10.5 Fiber Hygrometry Methods

Human hair and some synthetic fibers are hygroscopic and stretch when hydrated. By securing one end of a plastic thread and attaching the other end to a lever, the relative humidity of an atmosphere surrounding the thread can be read from a dial. To determine the a_w of a food, this device, which may include a capillary thermometer for temperature compensation, is incorporated into the lid of a canister in which the sample is held. Equilibration between sample and thread takes about three hours. The instrument should be calibrated weekly against saturated salt solutions. It is inexpensive and widely used for routine examinations in the food industry.[120] It is accurate to ± 0.01 a_w.

7.10.6 Electric Hygrometry Methods

Hygroscopic material in the form of an immobilized salt, usually LiCl, is held in a small chamber with a sample of food. The electrical conductivity of the material, when it and the food are in equilibrium, is related to its degree of hydration and hence to the ERH of the head space and the a_w of the sample.[189] The conductivity is measured and converted to a_w, which is displayed and recorded.

In spite of relatively high cost, electric hygrometers are the most commonly used instruments to measure a_w in both research and process control. The sensors need routine calibration with saturated salts in the a_w range relevant to the foods being examined. A disadvantage is the tendency for sensors to become contaminated with polar compounds such as glycerol, glycols, and volatile amines, which influences results. Filters may be used to absorb these compounds, but equilibration time is extended. Resting at low humidity and recalibration restores accuracy.[164,219]

7.10.7 Microthermometry

A small drop of a reference salt solution on the tip of a thermocouple is brought into contact with the atmosphere in water vapor equilibration with a sample. Depending upon the a_w of the sample, water vapor either evaporates from the drop or condenses onto it, and the temperature of the reference solution either falls or rises. The temperature change is measured by thermocouple and depends upon the difference between sample and reference a_w values. Response is linear from 0.78 to 1.00 a_w. The thermocouple is readily rinsed clean of any pollutants between readings; it has long-term stability, and there is no hysteresis. The accuracy is reported as ±0.0034 at 0.92 a_w and ±0.0053 at 1.00 a_w.[191] Another method based on the psychrometric consequences of water condensation onto a thermocouple has been described.[238]

7.11 MOISTURE DETERMINATION

In the construction of water sorption isotherms, accurate and reproducible determinations of moisture content are essential. A range of direct and indirect techniques for measuring the moisture content of foods has been reviewed.[105] The methods commonly employed in food laboratories involve drying weighed samples to constant weight over a desiccant (phosphorus pentoxide) or in ovens. The latter may be conventional, vacuum, infrared, or microwave. When dry weight is determined to establish the a_w of a sample by reference to a water sorption isotherm, it should be obtained by precisely the same method as that used to construct that isotherm. If time is less important than accuracy, conventional oven drying is recommended.[48] The microwave method is the best substitute for the conventional oven if time is important, but extreme care is necessary to standardize power level and drying time, taking into account the possibility of charring.[48]

7.12 CONCLUDING REMARKS

Drying and reduction of a_w are of major importance in food preservation. The most important contributors to the human diet, the cereal grains, are stabilized by low water content. In addition to preservation, drying of many foods bestows the benefits of greatly reduced mass and volume. Many of the traditional methods of preservation by reduced a_w persist, and important benchmarks appear to have been established: no microbial growth below 0.60 a_w, no mycotoxin production below ~0.80 a_w, no growth of pathogenic bacteria below ~0.85 a_w. How stable these criteria are remains to be seen.

To date, microbiologists have concentrated on a_w as the criterion most appropriate to define the availability to microorganisms of water in foods. It is not ideal, as solute specific effects exist. However, many of these instances of solute specificity are observed in laboratory media with solutes not present in foods at levels of significance to a_w. Attempts to explain these anomalies solely in terms of phase transition criteria have not proven helpful, although such criteria are probably more useful than a_w in defining textural properties of foods. Some as yet undetermined combination of these two approaches might greatly advance understanding of the properties of water in foods.

There is often virtue in reducing dependence on a sole inhibitory or preservative factor in foods and much attention is being given to preservation at higher levels of a_w in combination with such microbiological hurdles as reduced pH, mild heating, refrigeration, and chemical preservatives. Predictive microbiology is accumulating data that will facilitate the safe use of such combination treatments. Research on osmoregulation in microorganisms is defining the physiological and genetic basis for growth at low a_w and this may yield means to interfere with the homeostatic mechanisms involved and so improve the efficacy of reduced a_w preservation systems.

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8 Control of pH and Use of Organic Acids

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CHAPTER CONTENTS

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8.1 INTRODUCTION

The pH of foods influences their susceptibility to microbial growth, and acidification, either by the addition of acids or by fermentation, is used in preservation of many types of food. The approximate pH of a range of foods is shown in Exhibit 8-1.The acidity in meat and poultry is due largely to lactic acid, that of fermented dairy products is due also largely to lactic together with acetic acid and, in the case of a cheese such as Emmentaler, propionic acid. Acids such as malic, citric, and tartaric contribute to the acidity of fruits and vegetables. Acidulants used to reduce the pH of manufactured foods such as soft drinks include inorganic acids such as phosphoric acid. pH values of some acidified drinks are as low as 2. Alkaline pH is not used commercially to preserve foods, although a natural food such as egg white may have a pH of 9 or above, which can contribute to microbiological stability.

The optimum pH for growth of many food-associated microorganisms is in the range 6.5–7.5, but some can multiply in highly acidic conditions (Table 8-1). In general, yeasts and molds show a greater ability than bacteria to grow at acid pH, nevertheless some bacteria cause spoilage of acid foods.

Some acids are added to foods in order to adjust their acidity (acidulants or acidity regulators); others are added not to adjust the acidity but to act as preservatives based on their antimicrobial effect. Strong acids are those that are virtually completely dissociated in dilute aqueous solutions; of these, phosphoric acid is used in foods, mainly in cola-type drinks.[48] The remaining acidulants are weak organic acids, which have a range of strengths that are indicated by their dissociation constants, K_a or by pK_a, which is the negative logarithm of K_a. The weak organic acids that are used as preservatives include benzoic, sorbic, and propionic acids. The esters of hydroxybenzoic acid are also considered in this chapter. Some of the properties and applications of acids and esters that are used in foods are shown in Table 8-2. The concentrations of these compounds that may be added to foods are specified by legislation in many countries, but within these specifications the concentration used may be limited by the effect of the acid on the taste of the food.

The antimicrobial effect of weak organic acids used as acidulants is due partly to their influence on the pH of foods and partly to the specific effect of the

Exhibit 8-1 Approximate pH of a Range of Foods

Product	*pH*
Meat	
Beef (ground)	5.1–6.2[a]
Ham	5.9–6.1[a]
Veal	6.0[a]
Chicken	6.2–6.4[a]
Fish and Shellfish	
Fish (most species)	6.6–6.8[a]
White fish	5.5[a]
Salmon	6.1–6.3[a]
Crabs	7.0[a]
Tuna	5.2–6.1[a]
Vegetables	
Most green vegetables	4.5–7.1[b]
Asparagus	5.0–6.1[b]
Beans (lima)	5.4–6.2[b]
Cabbage (green)	5.2–6.3[b]
Carrots	4.9–6.3[b]
Potatoes	5.6–6.2[b]
Sweet potatoes	5.3–5.6[b]
Peppers (several types)	5.0–7.0[b]
Tomatoes, ripe	3.4–4.9[b]
Fruits[g]	
Apples	2.9–3.3, 3.3–4.1
Melons, cantaloupe	6.2–6.5
Melons, honeydew	6.3–6.7
Oranges, juice	3.6–4.3
Miscellaneous	
Mushrooms	6.0–6.5[c]
Dairy Products	
Milk	6.3–6.5[a]
Cream	6.5[a]
Butter	6.1–6.4[a]
Cultured buttermilk	4.6–4.7[e]
Cheese (Cheddar type)	5.2[f]
Cheese, Camembert, ripe	surface 7.0[d] center 6.0[d]
Cottage cheese	4.1–5.4[c]
Yogurt	4.6–5.0[e]

[a] From Jay.[61]
[b] From Lund.[78]
[c] From Banwart.[3]
[d] From Gripon.[53]
[e] From ICMSF.[60]
[f] See Chapter 23.
[g] See Chapter 27.

organic acid itself. This latter effect, and that of benzoic, sorbic, and propionic acids, is due mainly to the undissociated form of the acid. On the basis of the Henderson-Hasselbalch equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log([\mathrm{A}^-]/[\mathrm{HA}])$$

where HA is the acid and the anion A^- is the conjugate base, the percentage of undissociated acid at a given pH can be calculated from the formula:

$$[\mathrm{HA}]\% = 100/(1 + 10^{(\mathrm{pH} - \mathrm{p}K_a)})$$

Weak organic acids are valuable agents in food preservation but many microorganisms are able, in certain conditions, to metabolize subinhibitory concentrations of these acids. This ability may be accompanied by relatively high resistance to the acids. Resistance may also occur in organisms that are not able to metabolize the acid concerned and may be induced by growth in, or exposure to, sublethal concentrations.

The extent of the effect of pH and acidulants on microorganisms in foods may be modified by other components in the food, which may have additive or synergistic effects or, conversely, may protect microorganisms and may also influence the dissociation of organic acids. In foods with a high content of fat the concentration of benzoic and sorbic acids in the aqueous phase, where microorganisms are located, will be reduced because the solubility of the acid in fat is much greater than that in water. The partitioning between the water and fat phases of a food will depend on the pH, the type and amount of fat, and the concentration of other components such as sugars and salts.[101]

Information on the effect of pH and weak organic acids on microorganisms in culture media and in foods has been reviewed extensively.[21,33,98,101] The purpose of this chapter is to highlight main principles underlying the effect of these factors.

8.2 THE EFFECT OF pH, ACIDULANTS, AND ORGANIC ACID PRESERVATIVES ON GROWTH OF MICROORGANISMS

8.2.1 Investigation of the Effect of pH, Acidulants, and Preservatives on Growth

In order to investigate the effect of pH *per se* on microorganisms, in particular on foodborne pathogens, experiments have been done using nutrient media adjusted to the required pH with a strong acid, such as HCl. Because this acid is completely dissoci-

Table 8-1 Approximate pH Range for Growth of Microorganisms Important in Relation to Foods

Microorganism	*Min. pH*	*Opt. pH*	*Max. pH*	*Reference*
Bacteria (most)	4.5	6.5–7.5	9.0	3
Nonpathogenic bacteria				
Acetobacter spp.	3.0	5.0–6.0	—	63
Alicyclobacillus	2.0	3.5–4.0	7.0	104
Bacillus acidocaldarius	2.5	3.4–4.0	5.5	20
Clostridium butyricum (nontoxigenic)	4.2	—	—	85
Clostridium pasteurianum	3.55	—	—	109
	4.2	—	—	102
Enterobacter agglomerans	3.6	—	—	11
Escherichia coli (nonpathogenic)	4.0	—	—	94, 95
Gluconobacter spp.	3.0	5.5–6.0	—	75
	<3.3	—	—	40
Lactobacillus acetotolerans	3.3	—	6.3–6.8	38
Lactobacillus brevis	3.2	—	—	30
	3.0	—	—	62
Lactobacillus fructivorans	2.9–3.5	—	—	7, 30
Lactobacillus plantarum	<3.0	—	—	81
Pseudomonas fluorescens and *P. putida*	4.4	—	—	11
Pathogenic bacteria				
Bacillus cereus	5.0	6.0–7.0	8.8	59
Campylobacter jejuni/coli	4.9	6.5–7.5	ca. 9	59
Clostridium botulinum	4.6	—	—	59
Clostridium perfringens	5.0	—	9.0	(Chapter 42)
Escherichia coli (intestinally pathogenic)	4.4	6–7	9.0	59
Listeria monocytogenes	4.3–4.5	7	9.4	46, 59
Salmonella spp.	3.8	7–7.5	9.5	41, 59
Salmonella typhimurium	<4.0	—	—	73
Shigella flexneri	5.0	—	9.19	59
	4.7	—	—	73
Shigella sonnei	4.9	—	9.34	59
Staphylococcus aureus	4.0	6–7	10	59
Vibrio cholerae	5.0	7.6	9.6	59
Vibrio parahaemolyticus	4.8	7.8–8.6	11	59
Vibrio vulnificus	5.0	7.8	10	59
Yersinia enterocolitica	4.2	~7.2	9.6–10.0	59
Yeasts (general)	1.5–3.5	4.5–6.8	8.0–8.9	3
Candida krusei	1.3	—	—	91
Saccharomyces cerevisiae	1.6	—	—	91
Pichia membranifaciens	1.9	—	—	91
Zygosaccharomyces bailii	2.2–2.5	—	>7	91
	<2	—	—	108
	—	—	5.5	117
Molds (general)	1.5–3.5	4.5–6.8	8.0–11	3
Moniliella acetoabutans	<3.3	—	—	29
Aspergillus flavus, A. parasiticus, A. niger	2	5–8	>11	59, 92, 122
Fusarium equiseti	<3.3	5.0–8.0	>10.4	92, 122
Penicillium verrucosum	<2.1	6.0–7.0	>10.0	92, 122

Table 8-2 Acids and Esters Used as Acidulants (Acidity Regulators) or As Preservatives in Foods

Acid	*Formula*	*pK_a at 25°C*	*Typical Maximum Use Concentration (mg kg^{-1} or mg l^{-1})*	*Examples of Uses*
Acidulants				
Acetic acid	CH_3COOH	4.76^a	(i) generally permitted (ii) GRAS*	pickles, salad cream
Adipic acid	$HOOC.CH_2.CH_2.COOH$	(1) 4.43^a (2) 5.41^a	(i) 1000–6000 (ii) GRAS**	gel-like desserts, fruit-flavored desserts
Citric acid	$(COOH)CH_2.C(OH)(COOH).CH_2COOH$	(1) 3.13^b (2) 4.76^b (3) 6.40^b	(i) generally permitted (ii) GRAS***	soft drinks, fruit drinks, jams, preserves, processed cheese, canned fruit
Fumaric acid	$HOOC.CH{=}CH.COOH$	(1) 3.03^b (2) 4.54^b	(i) 4000	gel-like desserts, fruit-flavored desserts
Gluconic acid	$HOOC.CH(OH)CH(OH)CH(OH)CH(OH)CH_2OH$	3.6^b	(i) generally permitted	
Lactic acid (DL)	$CH_3.CH(OH).COOH$	3.86^b	(i) generally permitted (ii) GRAS*	salad dressing, soft margarine, soft drinks
Malic acid (DL)	$HOOC.CH(OH).CH_2.COOH$	(1) 3.40^a (2) 5.11^a		sometimes used with citric acid in soft drinks, jams
Succinic acid	$HOOC.CH_2.CH_2.COOH$	(1) 4.16^a (2) 5.61^a	(i) 5000–6000 (ii) GRAS*	desserts, soups, broths
Tartaric acid (L)	$HOOC.CH(OH).CH(OH).COOH.H_2O$	(1) 2.98^b (2) 4.34^b		juice-based drinks, jams
Phosphoric acid	H_3PO_4	(1) 2.16^a (2) 7.21^a (3) 12.32^a	700	cola-type, nonfruit drinks
Preservatives				
Propionic acid	$CH_3.CH_2.COOH$	4.86^a	(i) 1000–3000	prepacked bread, Christmas pudding
			(ii) GRAS	
			(ii) 3000	cheese products
			(ii) 3200	flour, white bread, and rolls
			(ii) 3800	whole wheat
				present naturally at up to 1000 mg kg^{-1} in Swiss cheese

continues

Table 8-2 Continued

Acid	*Formula*	*pK_a at 25°C*	*Typical Maximum Use Concentration (mg kg^{-1} or mg l^{-1})*	*Examples of Uses*
Benzoic acid	C_6H_5COOH	4.19[a]	(i) 1000	reduced-sugar jams, nonemulsified sauces, fruit-based drinks
			(i) 2000	semi-preserved fish products; cooked shrimps
			(i) 5000	liquid egg
			(i) 6000	*Crangon crangon* (shrimp) and *Crangon vulgaris,* cooked
			(ii) GRAS up to 1000	
Sorbic acid	$CH_3.CH=CH-CH=CH.COOH$	4.76[b]	(i) 1000	cheese, prepacked, sliced; unripened cheese; meat pate; low sugar jams
			(i) 2000	processed cheese; emulsified fats and sauces with <60% fat; semi-preserved fish products; cooked shrimps
			(i) 5000	liquid egg
			(i) 6000	*Crangon crangon* and *Crangon vulgaris,* cooked
			GRAS maximum level specified in many foods, usually 1000–2000 but 3000 in regular cheese	
Methyl, ethyl and propyl-hydroxybenzoates	$C_6H_5COOCH_3$ (methyl)	*ca.* 8.5[c]	(i) 1000	meat pate, jelly coatings of meat, liquid dietary supplements
			(i) 2000	
			(ii) GRAS up to 1000	

Organic acids used as preservatives are very often added as their salts.

[a] Data from Lide.[70]

[b] Data from Budavari.[16]

[c] Data from Foegeding and Busta.[42]

(i) Examples of limits taken from UK legislation.[82,105] These regulations implement European legislation.[39] Generally permitted means that no maximum level is specified, but the additive may be used in accordance with good manufacturing practice. For further information EC regulations and those for specific countries should be consulted.

(ii) Regulations in US. *GRAS for miscellaneous and general purpose usage, **GRAS when used as a buffer or neutralizing agent not to exceed good manufacturing practices, ***GRAS for miscellaneous and general purpose usage when used in accordance with good manufacturing practices.[23]

ated at pH values above about 2.0, adjustment of the pH with HCl enables the effect of hydrogen ions on microorganisms to be tested without the additional effect of an undissociated acid. In experiments to test the effect of pH, or to test the effect of other inhibitory factors, it is important to provide optimum nutrient conditions if the full extent of the ability of the organism to tolerate the inhibitory factor under investigation is to be determined. For example, tryptone soya medium plus yeast extract and glucose gave shorter lag times and more rapid growth of *Listeria monocytogenes* at pH 4.5 than tryptone phosphate medium.[45] pH influences (1) the probability of growth of microbes, (2) the rate of growth, and (3) survival and rate of death.

Many studies of the effect of pH and of organic acids on the ability of microorganisms to initiate growth use a standard inoculum into tubes or flasks or wells in microwell plates, containing medium adjusted to the required composition. Determinations of minimum inhibitory concentration (MIC) of organic acids and other preservatives are made in this way. Such experiments enable investigation of a wide range of experimental conditions but do not reveal the fact that the proportion of microorganisms in the inoculum that are able to initiate growth in different sets of conditions can differ considerably. Thus the MIC will depend on the number of viable organisms in the inoculum. The MIC does not include a measure of the time required for growth, which is often increased in the presence of an inhibitor; this can be reported as additional information. Thus, the inhibitory effects taking place are only partly analyzed in such experiments.

In the use of microwell plates, technical problems can arise that need to be prevented in order to avoid spurious results. With some types of microwell plate and lid evaporation tends to take place, resulting in concentration of solutes. To avoid this, the plates should be incubated at a very high humidity. Volatile compounds, such as acetic acid, may be lost during incubation for long periods of time. In addition, growth of microorganisms in subinhibitory conditions can result in release of volatile compounds, such as ammonia, that can alter the pH in adjacent wells[11] and so lead to spurious results. Measures need to be taken to avoid such changes.

8.2.2 The Effect of pH on Initiation of Growth

The results of experiments to determine the ability of microorganisms to initiate growth at acid pH depend on the number of the microorganisms in the inoculum. In demonstrating this, it is necessary to ensure that the inoculum itself does not change the pH of the medium. Two factors may contribute to the effect of number of microbes in the inoculum. First, there is a random chance that hydrogen ions or undissociated acid molecules will interact with the microbes. Second, the resistance of microorganisms and their ability to survive and multiply in adverse conditions may show a statistical distribution throughout the population, in which case increasing the number of microbes present will increase the likelihood that some will be present that are able to tolerate a given set of conditions. This is illustrated by experiments to test the ability of proteolytic, Group I *Clostridium botulinum* to multiply in a medium acidified with HCl. In conditions that were otherwise highly favorable, the probability of growth from single vegetative bacteria at pH 6.8 within 3 days at 30°C was 1 (ie, 100%), thus growth could occur from an inoculum of a single bacterium, whereas at pH 4.60–4.61, the probability of growth within 14 days was reduced to about 10^{-6} or 10^{-7}, so that an inoculum of between 10^6 and 10^7 bacteria would be required in order that one bacterium would be present that could initiate growth in these conditions (Figure 8-1).[80]

8.2.3 The Effect of pH on Rate of Growth and Yield

In addition to affecting the initiation of growth, pH also affects lag time and the rate of microbial growth. The effect of pH on the lag time will depend on the previous history of the inoculum, for example, on factors such as the phase of growth of the organism, the growth medium, the pH, and the temperature.[45] The effect of pH on growth rate is illustrated by a study of the growth of nonpathogenic *Escherichia coli* in medium adjusted with HCl to pH values between pH 7.0 and pH 4.0; an approximately linear inverse relationship was found between growth rate and hydrogen ion concentration (Figure 8-2).[94] As the pH is reduced, so the yield of cell mass per unit of substrate used falls, reflecting the increased energy needed to maintain a higher and near-constant cytoplasmic pH (see Section 8-4).

8.2.4 The Effect of Acidulants and Propionic Acid on Growth

Many studies have been made of the effect of acidulants on microbial growth by adjustment of the pH of the culture medium with weak organic acids. For example, in experiments to determine the mini-

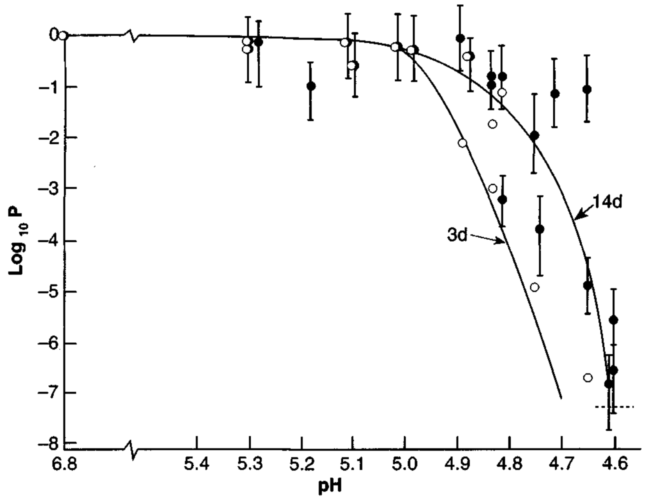

Figure 8-1 Effect of pH on the Probability (*P*) of Growth from Single Vegetative Bacteria of Proteolytic *Clostridium botulinum* ZK3 within 3 Days and 14 Days at 30°C. Continuous lines were predicted from a model based on results for this strain. Symbols represent experimental results for this strain: ○, after incubation for 3 days; ●, after incubation for 14 days. Bars represent 95% confidence limits of log P after 14 days; confidence limits for log P after 3 days were similar but are omitted for reasons of clarity. *Source:* Reprinted with permission from B.M. Lund, A.F. Graham, and J.G. Franklin, The Effect of Acid pH on the Probability of Growth of Proteolytic Strains of *Clostridium Botulinum, International Journal of Food Microbiology,* Vol. 4, pp. 215–226, © 1987, Elsevier Applied Science.

mum pH at which three serotypes of *Salmonella* would initiate growth in a medium adjusted to pH with a range of acids, the order of increase in reported inhibitory effect of these acids was very similar to the order of decrease of their lowest pK_a values (Table 8-3).[22] The concentration of acids required to adjust the pH was not reported.

Although such experiments simulate the practical use of acidulants, they do not allow an analysis of their effect, because they do not separate the influence of pH from that of the organic acid. In addition, the concentration of acidulant needed to adjust a medium or food to a required pH will differ according to the composition and buffering capacity of the medium or food. While an experiment using adjustment of pH with an acidulant will give useful information, the data may not be applicable directly to other media or foods.

In order to separate the effects of pH and of organic acids, the required concentration of the organic acid is added to the culture medium and the pH is adjusted to a specified value, eg, by the addition of HCl

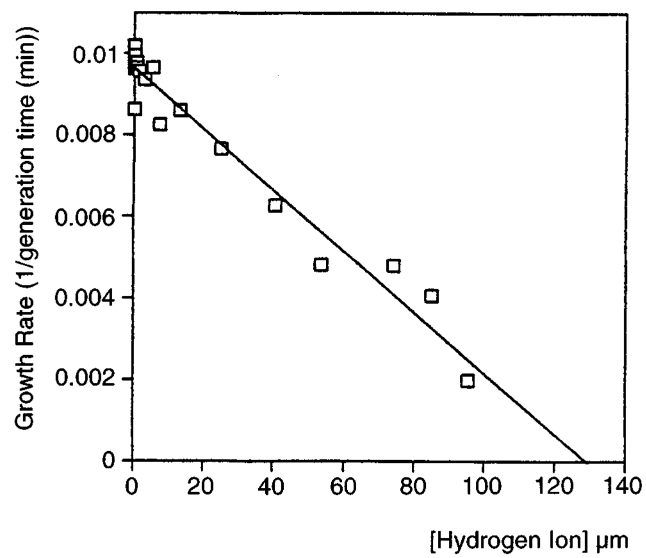

Figure 8-2 Effect of Hydrogen Ion Concentration, Adjusted with HCl, in Nutrient Broth on the Growth Rate of *Escherichia coli* M23 at 20°–22°C. The line was obtained by linear regression. *Source:* Reprinted with permission from K.A. Presser, D.A. Ratkowsky, and T. Ross, Modelling the Growth Rate of *Escherichia Coli* as a Function of pH and Lactic Acid Concentration, *Applied and Environmental Microbiology,* Vol. 63, pp. 2355–2360, © 1997, American Society for Microbiology.

Table 8-3 The Effect of Acidulants on the Minimum pH for Initiation of Growth of *Salmonella anatum, S. tennessee,* and *S. senftenberg* at 30°C from an Inoculum of 10^5 Bacteria, and Lowest pK_a Values for the Acids. The pH was adjusted with the acid being tested.

Acidulant	*Minimum pH for Growth*	*pK_a at 25°C*
Hydrochloric	4.05	0.00
Citric	4.05	3.13[a]
Tartaric	4.10	2.98[a]
Gluconic	4.20	3.60
Fumaric	4.30	3.03[a]
Malic	4.30	3.40[a]
Lactic	4.40	3.86
Succinic	4.60	4.16[a]
Glutaric	4.70	4.30
Adipic	5.10	4.43[a]
Pimelic	5.10	4.71
Acetic	5.40	4.76
Propionic	5.50	4.86

[a] Lowest pK_a value.

Source: Adapted with permission from K.C. Chung and J.M. Geopfert, Growth of *Salmonella* at Low pH, *Journal of Food Science,* Vol. 35, pp. 326–328, © 1970, Institute of Food Technologists.

and NaOH or KOH. An example of an experiment of this kind to show the effect of acidulants on initiation of growth of *Yersinia enterocolitica* is shown in Table 8-4. In these conditions up to 2% (w/v) citric acid had no significant effect on the pH at which growth occurred, and on the basis of total concentration, acetic acid was more inhibitory than lactic acid.[12] These experiments were done using microwell plates, with techniques to avoid changes in conditions in wells prior to growth.

Experiments using shaken tubes to study the effect of a series of concentrations of lactic acid adjusted to a specified pH with HCl or NaOH showed that between pH 4 and 7 in the presence of lactic acid, the reduction in growth rate of nonpathogenic *E. coli* was proportional to the concentration of undissociated lactic acid (Figure 8-3). For total lactic acid concentrations of 0–100 mM (ie, up to 0.9% w/v) the reduction of growth rate was equal for equal concentrations of undissociated acid, regardless of the pH or total lactic acid concentration.[94] The authors concluded that under the conditions normally present in foods, that is, at low concentrations of organic acid and low pH, the undissociated acid was the most significant inhibitory factor. However, they concluded that at high concentrations of lactic acid and high pH, the inhibitory effect of the dissociated acid may be significant. For this reason, they included the dissociated acid in the mathematical model that they developed to describe the effects of pH and lactic acid. At temperatures from 10° to 37°C and pH values up to 7.0, the concentrations of undissociated lactic acid that prevented growth were between 5.75 and 8.4 mM.[95]

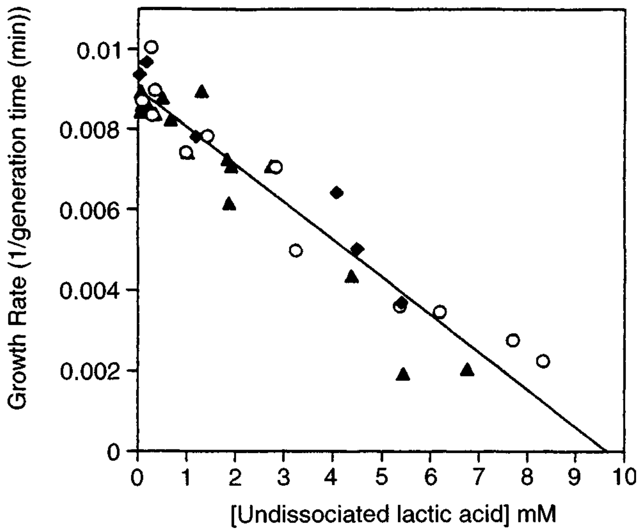

Figure 8-3 Effect of Concentration of Undissociated Lactic Acid in Nutrient Broth on the Growth Rate of *Escherichia coli* M23 at pH 4.0–6.0 and 20°–22°C. ◆, 25 mM; ○, 50 mM; ▲, 100 mM total lactic acid. Media were adjusted to the required pH with HCl and NaOH. *Source:* Reprinted with permission from K.A. Presser, D.A. Ratkowsky, and T. Ross, Modelling the Growth Rate of *Escherichia Coli* as a Function of pH and Lactic Acid Concentration, *Applied and Environmental Microbiology,* Vol. 63, pp. 2355–2360, © 1997, American Society for Microbiology.

Table 8-4 The Effect of Organic Acids on the Minimum pH, Adjusted with HCl, That Allowed Growth of *Yersinia enterocolitica*, Serotype 0:9. Inoculum approx. 2.5 × 10^4 bacteria in 250 µl medium per well of a microplate, incubation at 20°C for 8 days.

Additional Acid (pH adjusted with HCl or NaOH)	*Concentration*		*Minimum pH for Growth*
	%(w/v)	*mM*	
None			4.36–4.28
Acetic	0.1	17	4.36–4.28
	0.2	33	5.55–5.40
	0.3	50	5.72–5.51
Citric	0.5	24	4.38–4.17
	1	48	4.56–4.36
	2	95	4.55–4.36
Lactic	0.2	22	4.82–4.39
	0.4	44	5.22–4.81
	1	111	5.58–5.22

Source: Adapted with permission from T.F. Brocklehurst and B.M. Lund, The Influence of pH, Temperature and Organic Acids on the Initiation of Growth of *Yersinia Enterocolitica, Journal of Applied Bacteriology,* Vol. 69, pp. 390–397, © 1990, Blackwell Science, Ltd.

Similar types of experiments were used to study the effect of acetic and lactic acids on growth of *L. monocytogenes*.[46] The experiments used an optimal culture medium and good aeration, and started with a relatively low inoculum (10^2–10^3 bacteria ml^{-1}). This enabled measurement of the growth rate before changes in the culture medium, due to products of metabolism and limitation of available oxygen, would be sufficient to limit the growth rate. The effects of pH between 4.5 and 7.0 and of total acetic acid or lactic acid concentrations of 2,500 mg l^{-1} and 5,000 mg l^{-1} on the specific growth rate (µ) of *L. monocytogenes* are shown in Figure 8-4. The effect of a specific concentration of acid increased as the pH decreased, ie, the ratio (µ acidulant)/(µ HCl) decreased as the pH decreased, and the effect of acetic acid was greater than that of a similar concentration

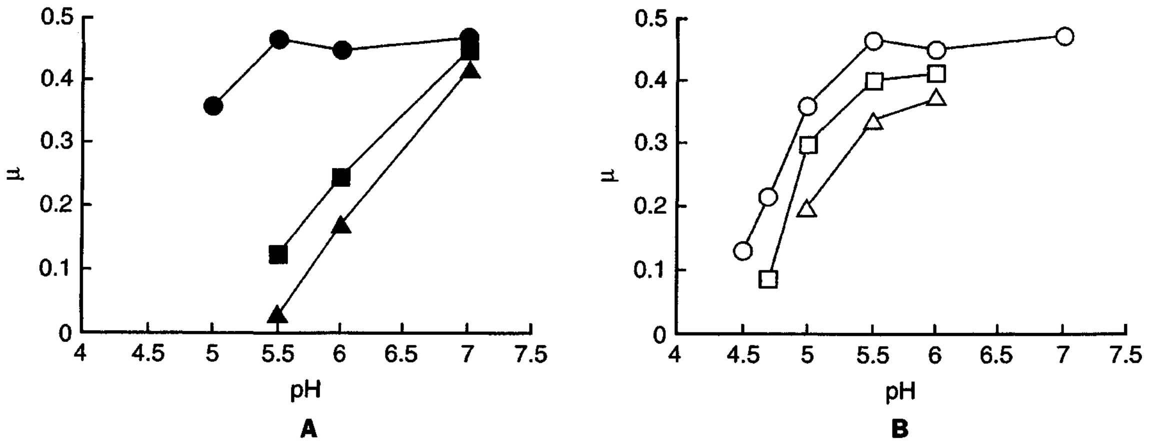

Figure 8-4 (**A**) Effect of pH and Total Acetic Acid on the Specific Growth Rate, μ, of *Listeria monocytogenes* F6861, Serotype 4b at 20°C. Culture medium: tryptone soya broth plus yeast extract, 3 g l^{-1}, and glucose, 10 g l^{-1}, pH adjusted with KOH or HCl. ●, no added acetic acid; ■, 2,500 mg acetic acid l^{-1}; ▲, 5,000 mg acetic acid l^{-1}. (**B**) Effect of pH and Total Lactic Acid on the Specific Growth Rate, μ, of *Listeria monocytogenes* F6861, Serotype 4b at 20°C. Culture medium as in Figure 8-4 (**A**). ○, no added lactic acid; □, 2,500 mg lactic acid l^{-1}; △, 5,000 mg lactic acid l^{-1}. *Source:* Data from S.M. George, L.C.C. Richardson, and M.W. Peck, Predictive Models of the Effect of Temperature, pH and Acetic and Lactic Acid on the Growth of *Listeria Monocytogenes, Food Microbiology,* Vol. 32, pp. 73–90, © 1996, Academic Press, Ltd.

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of lactic acid. On the basis of the effect of total concentration of acid, expressed as mg l^{-1} (Figure 8-5A) or as mM, on growth rate at pH 5.0, acetic acid had a greater effect than lactic acid, but on the basis of the calculated concentrations of undissociated acid, expressed in mM, limited experiments indicated that the effect of the two acids was similar (Figure 8-5B).

The relative effect of acidulants on different microorganisms may depend on differences in the ability of these microbes to utilize the acids. Of the acidulants that are used widely in foods, acetic acid is usually found to have the greatest antimicrobial effect when assessed on the basis of total concentration, which would be expected from its pK$_a$ value.

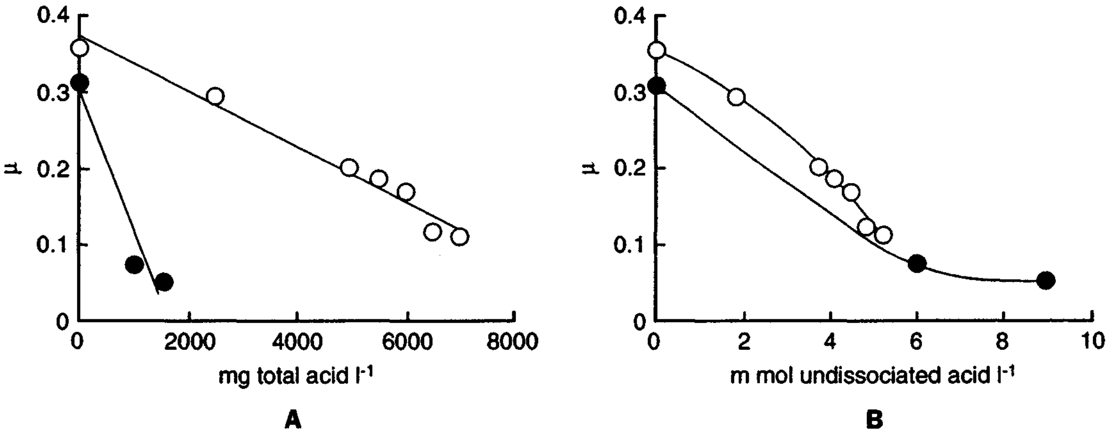

Figure 8-5 (**A**) The Effect of Total Acetic Acid and of Total Lactic Acid, mg l^{-1}, on Specific Growth Rate, μ, of *Listeria monocytogenes* F6861 at pH 5.0 and 20°C. Culture medium as in Figure 8-4 (**A**). ●, acetic acid; ○, lactic acid. (**B**) The Effect of Undissociated Acetic Acid and of Undissociated Lactic Acid, m mol l^{-1}, on Specific Growth Rate, μ, of *Listeria monocytogenes* F6861 at pH 5.0 and 20°C. Culture medium as in Figure 8-4 (**A**). ●, acetic acid; ○, lactic acid. *Source:* Data from S.M. George, L.C.C. Richardson, and M.W. Peck, Predictive Models of the Effect of Temperature, pH and Acetic and Lactic Acid on the Growth of *Listeria Monocytogenes, Food Microbiology,* Vol. 32, pp. 73–90, © 1996, Academic Press, Ltd.

For sensory reasons, the concentration of acetic acid used in foods may be limited, and it may be used in combination with other acidulants such as lactic and citric acid.

Adaptation of *Salmonella* species to growth at lower pH, adjusted with lactic acid, has been reported.[58] Growth on the surface of an agar medium containing a graded concentration of lactic acid, resulting in a pH gradient, reduced the growth-limiting pH for *Salmonella madelia* in a liquid medium, acidified with lactic acid, from 5.2 to 4.6. For other serovars the reduction in growth-limiting pH was about 0.8 units. This increased ability to multiply at acid pH was lost quickly, ie, after two transfers on tryptic soy agar plates.

In experiments described above, acetic acid concentrations of the order of 0.5% caused a marked decrease in the growth rate of *L. monocytogenes* at pH 5.5. In contrast, some bacteria can tolerate much higher concentrations of acetic acid. For example, *Lactobacillus* species can be important in spoilage of acetic acid preserves and *L. fructivorans*, *L. buchneri*, *L. brevis*, and *L. casei* were reported to tolerate acetic acid concentrations of up to 4.2%, 2.5%, 2.5%, and 1% respectively.[29,111] *Lactobacillus fructivorans* caused spoilage of tomato ketchup that had a pH of 3.8 and contained acetic acid and benzoic acid.[7] *Lactobacillus acetotolerans*, isolated from fermented broth of rice vinegar, could multiply in 9% to 11% acetic acid at pH 5.0 and in 4% to 5% acetic acid at pH 3.5.[38]

The yeasts *Saccharomyces acidifaciens* (probably synonymous with *Zygosaccharomyces bailii*[66,113]) and to a lesser extent *Pichia membranifaciens* have been reported as major causes of spoilage of acetic acid preserves in the United Kingdom (UK)[28] and have been reported to tolerate 2.5% to 3.0% acetic acid.[24,111] Yeasts were reported to cause sporadic spoilage of sauces containing at least 1% acetic acid, and 0.7% to 1.0% acetic acid used to preserve tomato sauce was insufficient to give complete inhibition of all spoilage yeasts.[91] Even 2% acetic acid was considered to be inadequate to prevent spoilage of tomato sauce by *Z. bailii* and possibly by *P. membranifaciens*. It was concluded that the stability of the product must depend on aseptic packing and subsequent exclusion of spoilage yeasts.

In the pH range 3.0–3.5, citric, malic, and tartaric acids failed to show an inhibitory effect against spoilage yeasts.[91] *Saccharomyces uvarum*, *Geotrichum candidum*, *Endomycopsis burtonii*, and *Hansenula canadensis* all grew at pH 4.5 with 1% lactate as an energy source but not with 1% propionate as energy source.[83] In the presence of 2% glucose, these yeasts grew at pH 4.5 in the presence of 1% lactate or 1% acetate but not 1% propionate. At acid concentrations up to 400 mM, lactate had less inhibitory effect on growth rate than acetate, but propionate was much more inhibitory than either. As the pK_a values of acetic and propionic acids are similar the greater inhibitory effect of propionic acid indicated a difference between the mode of action of these two acids. This was correlated with utilization by these yeasts of some acetate at low and high concentrations but little or no utilization of propionate.

The mold *Moniliella acetoabutans* was reported as the occasional cause of severe spoilage of acetic acid preserves. It could cause major problems in pickle production, and was sometimes difficult to eradicate once established in a factory.[30,106] If oxygen was available, this mold had been found as the cause of spoilage of products containing >5% acetic acid, but evidence was reported that it could be inhibited by concentrations of sorbic acid that would be suitable for use in foods.[30] *M. acetoabutans* was reported to tolerate 8% to 9% acetic acid at pH 4.0, whereas *Monascus ruber* and *Penicillium glaucum* tolerated 1.8% and 1.4%, respectively.[111]

Acetic acid concentrations of 0.8% to 1.0% at pH 3.5 inhibited *Saccharomyces ellipsoides* and *Penicillium glaucum* and 4% acetic acid was required to give the same effect at pH 7.0.[27] At pH 3.5, 0.2% acetic acid inhibited a wide range of molds involved in the spoilage of bread.[64] At pH 4.5 and pH 5.0, sodium acetate concentrations of 1% and 2%, respectively, completely inhibited growth and aflatoxin production by *Aspergillus parasiticus* at 28°C, whereas concentrations of 0.6% or 0.8% gave partial inhibition and decreased aflatoxin production by 70% and 90%, respectively.[14] A further stain of *A. parasiticus* and six strains of *A. flavus* were inhibited similarly. Propionic acid was more inhibitory, 0.2% at pH 4.5 completely inhibiting growth of three strains of *A. parasiticus*.

Some weak organic acids can have a chelating effect and their antimicrobial activity may depend on the concentration of metal ions present. For example, in a medium adjusted to pH 5.2 with HCl, citric acid concentrations of 10–50 mM (0.2% to 1.0% w/v) inhibited growth from vegetative bacteria and from spores of proteolytic *Clostridium botulinum*, but the inhibition of vegetative bacteria could be prevented and the inhibition of spores reduced by the addition of 12 mM Ca^{2+}.[52] Inhibition of the vegetative bacteria by citric acid could also be prevented by the addition of Mg^{2+} and greatly reduced by Fe^{2+} or Mn^{2+}.

8.2.5 The Effect of Benzoic and Sorbic Acids on Growth

Benzoic acid and sorbic acids are most inhibitory at acid pH; benzoic acid particularly is used mainly to inhibit yeasts and molds in acidic foods. These acids are usually added as their more water-soluble salts, sodium benzoate and potassium sorbate. Several groups of fungi and bacteria, including *Aspergillus niger*, species of *Rhizopus* and *Mucor*, *Pseudomonas putida*, and *Bacillus* species, can metabolize benzoic acid[21]; some strains of the molds *Penicillium*, *Aspergillus*, *Fusarium*, *Mucor*, and *Geotrichum*, and some lactic acid bacteria can degrade sorbic acid.[101]

The reported MICs of benzoic acid at pH 2.6–5.0 against a range of fungi were between 20 and 2,000 mg l^{-1}.[21] Reducing the pH of the culture medium by adjustment with HCl gave a considerable reduction in the amount of potassium sorbate and of potassium benzoate required to inhibit *Aspergillus parasiticus*.[99] At pH 5.5 the maximum concentrations of potassium benzoate and of potassium sorbate that allowed growth were equivalent to 3,048 mg benzoic acid l^{-1} and 1,493 mg sorbic acid l^{-1}, respectively, whereas at pH 4.5 the maximum concentrations allowing growth were equivalent to 762 mg benzoic acid l^{-1} and 373 mg sorbic acid l^{-1}. Whereas some species of fungi do not grow in the presence of 3,000 mg sorbic acid l^{-1} at pH 5.5 and 28°C, others, in particular *Penicillium* species, are more resistant.[71,72] Sorbate-resistant strains of *P. roquefortii* grew at reduced rates in the presence of an initial concentration of up to 9,000 mg sorbic acid l^{-1}. These strains degraded sorbic acid to 1,3 pentadiene, a volatile compound with an unpleasant, kerosene-like odor.[72] In a study of 263 isolates of *Penicillium* spp. isolated from moldy surplus cheese, 57% grew at 25°C and pH 5.5 in medium containing 3,000 mg sorbic acid l^{-1}, 8.8% grew in medium containing 4,500 mg sorbic acid l^{-1}, 0.8% grew in the presence of 6,000 mg sorbic acid l^{-1}, and none grew in the presence of 9,000 mg sorbic acid l^{-1}.[110] In these conditions relatively high concentrations were required to inhibit the *Penicillium* spp., but reduction of the pH and reduction of temperature, conditions applying in many refrigerated foods, would be expected to lower the concentrations of sorbic acid required for inhibition.

The MIC of benzoic acid against a range of yeasts at pH 2.6–5.0 was between 20 and 700 mg l^{-1} but for *Zygosaccharomyces rouxii* and *Z. bailii* at pH 4.8 MICs of 1,000 and 4,500 mg l^{-1}, respectively, have been reported.[21] Resistance of yeasts to benzoic acid was correlated with resistance to sorbic acid[114] (Table 8-5).

Table 8-5 illustrates the difference in sensitivity of yeasts to these acids; it is notable that the strain of *Z. bailii* isolated from a winery was less resistant than the strains isolated from spoiled beverages containing benzoic acid, despite the fact that the inocula for the experiment were grown in the absence of preservative. Growth of *Z. bailii* in the presence of a low level of benzoic acid increased its resistance to this acid.[113] This effect was shown by a series of yeasts, including relatively sensitive strains (Table 8-6).[114] This adaptation of yeasts resulting in resistance to benzoic acid, was correlated with resistance to acetic and propionic acids but not to methyl paraben.[117]

For bacteria at pH 4.3–6.3 reported MICs of benzoic acid are generally between 50 and 3,000 mg l^{-1}, the highest values cited were against *Listeria monocytogenes* and *Lactobacillus* species.[21] In tryptose broth adjusted to pH 5.6 with HCl and incubated at 21°C, 2,000 mg sodium benzoate l^{-1}, equivalent to 1,695 mg benzoic acid l^{-1}, reduced the rate of growth of a strain of *L. monocytogenes* and 2,500 mg sodium benzoate l^{-1} prevented growth.[37] At pH 5.0 and 21°C 2,000 mg sodium benzoate l^{-1} prevented growth and lower concentrations reduced the rate of growth, whereas at pH 5.0 and 13°C 500 mg sodium benzoate l^{-1}, equivalent to 423 mg benzoic acid l^{-1}, prevented growth.

Reported MIC values of sorbic acid for several bacteria and for *Candida albicans* at 37°C, based on experiments in microwell plates, were generally much lower at more acidic pHs; examples of results are shown in Table 8-7.[34] *Staphylococcus aureus* was relatively resistant while *Pseudomonas aeruginosa* was reported to be extremely resistant. In these conditions some of the concentrations of sorbic acid used were much higher than would be used in most foods.

A similar pattern of sensitivity to benzoic acid was reported (Table 8-8).[35] Greater inhibition would be expected at lower temperatures.

Some bacteria are relatively resistant to benzoic and sorbic acid at an acidic pH, including some gluconobacters, which are bacteria that oxidize ethanol to acetic acid. In the case of a representative strain of *Gluconobacter oxydans* that had caused spoilage of a fruit product preserved with sorbic acid, the MICs of sorbic acid and benzoic acid at pH 3.8 and 25°C were 1,000 mg l^{-1} and 900 mg l^{-1}, respectively, and at pH 3.3 were 700 mg l^{-1} and 500 mg l^{-1}, respectively.[40] Growth of the organism in the presence of 400 mg benzoate or sorbate l^{-1} at pH 3.8 resulted in a considerable increase in the MIC and adaptation to increased resistance to sorbate or

Table 8-5 The Effect of Benzoic and Sorbic Acids on Anaerobic Growth of Yeasts at pH 3.5. Inoculum, grown in preservative-free medium, 10^4 cells per 5 ml medium; incubation at 25°C for 1,000 hours. (Sorbic acid, 1 mM = 112 mg l^{-1}; benzoic acid, 1 mM = 122 mg l^{-1}.)

	Sorbic Acid		*Benzoic Acid*	
Organism and FRR Number	*Maximum Concn. Allowing Growth (mM)*	*Minimum Concn. Preventing Growth (mM)*	*Maximum Concn. Allowing Growth (mM)*	*Minimum Concn. Preventing Growth (mM)*
Kloeckera apiculata 2168	1	1.4	1.5	2
Hansenula anomala 2169	1	1.5	0.5	1
Kluveromyces fragilis 1338	1	1.5	1	ND
Saccharomyces cerevisiae 1297[a]	1	ND	0.7	1
S. cerevisiae 1298[b]	2	3	2	3
Candida krusei 1302	3	ND	3	ND
Saccharomycodes ludwigii 1555[c]	3	4	3	3
Schizosaccharomyces pombe 2535[d]	4	6	4	6
Zygosaccharomyces bailii 2476[b]	2	3	2	3
Z. bailii 1292[d]	4	6	4	6
Z. bailii 2227[d]	4	6	4	6

[a] A baker's yeast.
[b] From a winery.
[c] From apple cider.
[d] Isolated from spoiled beverages containing benzoic acid.
ND, not detected.

Source: Adapted with permission from A.D. Warth, Resistance of Yeast Species to Benzoic Acids and Sulfur Dioxide, *Journal of Food Protection*, Vol. 48, pp. 564–569, © 1985, International Association of Milk, Food and Environmental Sanitarians, Inc.

benzoate took place within 1 hour at 25°C. In these conditions, however, *G. oxydans* did not degrade sorbic or benzoic acid at an appreciable rate. *G. oxydans* required highly aerobic conditions for good growth, and elimination of the head space of cultures combined with addition of 400 mg ascorbate and 400 mg sorbic acid l^{-1} to medium at pH 3.8 prevented growth. Sorbic acid–resistant strains of *G. oxydans* isolated from grapes were only slightly inhibited by 1,000 mg sorbic acid l^{-1} in apple juice, pH 3.4.[103]

The concentration of undissociated sorbic acid was correlated with the inhibitory effect of sorbic acid on *Clostridium botulinum*. An undissociated sorbic acid concentration of 28 mg l^{-1} at pH 6.82, equivalent to a total sorbic acid concentration of 3,169 mg l^{-1}, increased the time required for growth at 37°C from spores of three, proteolytic, Group I strains by a factor of between 1.2 and 1.8.[8] At pH 5.65 a total sorbic acid concentration of 2,600 mg l^{-1}, equivalent to an undissociated sorbic acid concentration of 250 mg l^{-1}, increased the time required for growth from an inoculum of about 5×10^4 spores of 10 proteolytic strains by factors of between 2 and 4 compared with the time required in the absence of sorbic acid. A total sorbic acid concentration of 2,000 mg l^{-1} caused little reduction in the probability of growth from vegetative bacteria of proteolytic *C. botulinum* at 30°C and pH 7.0 or 6.0 but at pH values of 5.5–4.9 sorbic acid was much more inhibitory (Table 8-9).[79] At all these pH values, a total sorbic acid concentration sufficient to give about 280 mg undissociated sorbic acid l^{-1} reduced the probability of growth from single vegetative bacteria by a factor greater than 10^4. In initial concentrations of sorbic acid that allowed growth the concentration of sorbic acid became depleted, probably because of degradation by *C. botulinum*. Some other bacteria, particularly lactic acid bacteria, can degrade sorbic acid, producing pungent compounds such as 2,4 hexadien-1-ol and 3,5 hexadien-2-ol.[71]

8.2.6 The Effect of Parahydroxybenzoates on Growth

The pK_a values of parahydroxybenzoates (parabens) are much higher than those of benzoic and sorbic acids (Table 8-2), and at neutral pH parabens have a greater antimicrobial effect than benzoic and sorbic acids against many microorganisms.

Inhibition of molds by parabens has been reported to increase with increase in length of the alkyl chain.[31] A similar effect has been reported against Gram-positive bacteria but has not been observed consistently against Gram-negative bacteria. The concentrations of methyl, ethyl, and propyl parabens required to give total inhibition of a range of fungi, including strains of *Aspergillus niger*, *Penicillium digitatum*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces bailii*, in various conditions of temperature, pH, and time of incubation were between 63 mg l^{-1} and 1,000 mg l^{-1}.[31]

The concentrations required to give total inhibition of a range of Gram-positive and Gram-negative bacteria in various conditions were between 40 mg l^{-1} and 4,000 mg l^{-1}.[31] At pH 6.0 the MICs of methyl paraben against food-associated bacteria were substantially lower than those of sorbic acid, except in the case of *Aeromonas hydrophila*, whereas at pH 5.0 the MICs were similar (Table 8-10).[83] At pH 6.0 and 5°C the growth of these four bacteria was inhibited by paraben concentrations below 1,000 mg l^{-1}, the approximate concentration generally permitted in foods. In the presence of these antimicrobials, the rate of growth of these bacteria was reduced.

Table 8-6 The Effect of Growth of Yeasts in a Subinhibitory Concentration of Benzoic Acid on Resistance to Benzoic Acid at pH 3.5. Inoculum 10^4 yeasts per 250 µl medium. Incubation at 25°C for 6 weeks.

Organism and FRR Number	*Minimum Inhibitory Concentration of Benzoic Acid (mg l^{-1})* *Yeasts Grown without Benzoic Acid*	 *Yeasts Grown with Benzoic Acid*
Saccharomyces cerevisiae 1297	100	170[a]
Kluveromyces fragilis 1338	125	173[a]
Kloeckera apiculata 2168	125	188[a]
Hansenula anomala 2169	140	223[a]
S. cerevisiae 1298	175	285[a]
Candida krusei 1302	300	440[b]
Saccharomycodes ludwigii 1555	300	650[b]
Schizosaccharomyces pombe 2535	325	567[b]
Zygosaccharomyces bailii 1292	600	1,250[b]

[a] Yeast grown overnight in the presence of 30 mg benzoic acid l^{-1}.

[b] Yeast grown overnight in the presence of 244 mg benzoic acid l^{-1}.

Source: Reprinted with permission from A.D. Warth, Effect of Benzoic Acid on Growth Yield of Yeasts Differing in Their Resistance to Preservatives, *Applied and Environmental Microbiology*, Vol. 54, pp. 2091–2095, © 1988, American Society for Microbiology.

8.3 THE EFFECT OF pH, ACIDULANTS, AND ORGANIC ACID PRESERVATIVES ON SURVIVAL AND DEATH OF MICROORGANISMS

Even when microorganisms are unable to multiply in acidic conditions they may be able to survive

Table 8-7 The Effect of pH on the Minimum Inhibitory Concentration (MIC) of Sorbic Acid against *Escherichia coli* W-23, *Pseudomonas aeruginosa* ATCC 9027-9, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* JSM 101. 50 µl of culture added to 200 µl medium adjusted to the required pH with HCl or KOH. Incubation at 37°C for 5 days.

		pH						
	MIC	*4.6*	*5.0*	*5.6*	*6.0*	*6.6*	*7.0*	*7.6*
E. coli	mM	4.3	6.5	8.7	24	39	54	87
	mg l^{-1}	*482*	*730*	*974*	*2,681*	*4,384*	*6,055*	*7,804*
P. aeruginosa	mM	13	28	63	87	102	122	141
	mg l^{-1}	*1,458*	*3,169*	*7,064*	*9,755*	*11,437*	*13,680*	*15,844*
S. aureus	mM	8.7	13	28.3	41.3	74	83	217
	mg l^{-1}	*975*	*1,462*	*3,169*	*4,631*	*8,286*	*9,262*	*24,377*
C. albicans	mM	0	6.5	8.7	10.9	15	13	24
	mg l^{-1}	*0*	*730*	*974*	*1,219*	*1,704*	*1,457*	*2,580*

Source: Calculated using data from T. Eklund, The Antimicrobial Effect of Dissociated and Undissociated Sorbic Acid at Different pH Levels, *Journal of Applied Bacteriology*, Vol. 54, pp. 383–389, © 1983, Blackwell Science, Ltd.

Table 8-8 The Effect of pH on the Minimum Inhibitory Concentration (MIC) of Benzoic Acid against *Escherichia coli* ML 308-225, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* JSM 101. 50 μl culture added to 200 μl medium adjusted to the required pH with HCl or KOH. Incubation at 37°C for 5 days.

		pH						
	MIC	*4.6*	*5.0*	*5.6*	*6.0*	*6.6*	*7.0*	*7.6*
E. coli	mM	8.3	16.7	25	41.7	58.3	83.3	87.5
	mg l^{-1}	*1,017*	*2,039*	*3,052*	*5,091*	*7,118*	*10,171*	*10,684*
S. aureus	mM	8.3	25	62.5	95.8	125	137.5	225
	mg l^{-1}	*1,017*	*3,052*	*7,631*	*11,697*	*15,262*	*16,788*	*27,472*
C. albicans	mM	4.2	12.5	29.2	33.3	58.3	66.6	91.6
	mg l^{-1}	*509*	*1,526*	*3,565*	*4,066*	*7,118*	*8,132*	*11,184*

Source: Calculated using data from T. Eklund, Inhibition of Microbial Growth at Different pH levels by Benzoic and Propionic Acids and Esters of p-Hydroxybenzoic Acid, *International Journal of Food Microbiology*, Vol. 2, pp. 159–167, © 1985, Elsevier Applied Science.

for a prolonged period of time. In the case of foodborne pathogens this may have important consequences for the safety of foods. For example, outbreaks of food poisoning have been attributed to the survival of *Salmonella* spp. in unpasteurized apple cider[17] and in orange juice,[26,86] and by the survival of *E. coli* O157 in unpasteurized apple juice and cider[6,18,19] (see Chapter 27).

Prior exposure of bacteria to low pH can result in increased survival in acid conditions. Because of outbreaks such as those cited above, this effect has been studied particularly in *Salmonella* spp., *E. coli,* and *Shigella.* Different groups of workers have used different experimental conditions to study response to low pH stress, which has resulted in the description of several types of phenomenon.[4]

The term exponential-phase (log-phase) acid tolerance has been described usually on the basis of experiments using cells grown in minimal glucose medium. If cells that are growing rapidly at pH 7.7 are shifted to a pH below 4, they die rapidly. If cells growing rapidly at pH 7.7 are exposed to pH 6 for one generation their ability to survive when exposed to pH 3 increases.This has been termed an inducible acid tolerance response (ATR). Acid tolerance has also been described in stationary-phase cells.[73]

The term acid resistance (AR) has been used in general to describe the results of measurements made on stationary-phase cells (rather than exponential-phase cells) grown in a complex medium and in which survival is measured at about pH 2.5.

The term acid habituation (AH) has been used to describe the response of exponential-phase *E. coli* grown in nutrient broth at pH 5.0. These so-called acid-habituated cells will survive better at pH 3.5 or 3.0 than cells grown at pH 7.0.[50]

The following examples illustrate increased survival of *S. typhimurium, E. coli,* and *L. monocytogenes* on exposure to acid stress. *S. typhimurium* LT2 (an attenuated strain[4]) showed several mechanisms of adaptation to survive exposure to acid con-

Table 8–9 Effect of Sorbic Acid and pH on the Log Probability (P)[a] of Growth from Single Vegetative Bacteria of *Clostridium botulinum* ZK3, Type A, in Peptone-Yeast-Glucose-Starch Medium in 14 days at 30°C

pH	*Total Sorbic Acid (mg l^{-1})*	*Undissociated Sorbic Acid (mg l^{-1})*[b]	*Log Probability (P) of Growth in 14 Days*		
			Expt 1	*Expt 2*	*Expt 3*
5.5	0	0	0	−0.34	−0.15
	1,015	156	−0.38	−1.25	−0.28
	1,219	188	NT[c]	NT	−1.08
	1,421	219	−3.23	−1.67	−3.34
	1,828	281	−5.53	−4.32	−5.65
5.1	0	0	−0.38	NT	−0.76
	500	156	−0.20	−2.20	−1.53
	600	188	NT	NT	−1.79
	700	219	−1.50	−3.56	−1.58
	900	282	−5.53	−5.34	−6.08
4.9	0	0	−0.53	−2.82	−1.20
	374	157	−0.28	−3.41	−1.40
	499	189	NT	NT	−1.82
	524	220	−4.53	−3.48	−6.00
	674	283	−6.38	−6.60	−7.49

[a] Log P calculated from: P = (MPN of bacteria determined in the test conditions)/(MPN of bacteria inoculated into the test conditions, determined in the medium at pH 6.8–7.0 without sorbic acid).

[b] Calculated.

[c] NT = not tested.

Source: Adapted with permission from B.M. Lund, S.M. George, and J.G. Franklin, Inhibition of Type A and Type B (Proteolytic) *Clostridium Botulinum* by Sorbic Acid, *Applied and Environmental Microbiology*, Vol. 53, pp. 935–941, © 1987, American Society for Microbiology.

Table 8-10 The Effect of pH, Adjusted with HCl and NaOH, on the Minimum Inhibitory Concentration (MIC) of Methyl Paraben and Potassium Sorbate against Food-Associated Bacteria. Inoculum 10^3 bacteria in 5.5 ml medium.

		MIC (mg l^{-1})							
		30°C				*5°C*			
		pH 5		*pH 6*		*pH 5*		*pH 6*	
	Buffer[a]	*Paraben*	*Sorbate*	*Paraben*	*Sorbate*	*Paraben*	*Sorbate*	*Paraben*	*Sorbate*
Listeria monocytogenes	C	300	400	1,300	>6,000	NT[b]	NT	NT	NT
	P	700	600	1,600	>5,000	—[c]	—	600	1,500
Yersinia enterocolitica	C	250	200	350	1,600	50	100	100	1,100
Aeromonas hydrophila	P	200	50	550	550	—	—	100	150
Pseudomonas putida	C	300	250	450	2,800	550	450	550	>5,000

[a] C = citrate/phosphate buffer, P = phosphate buffer.

[b] NT = not tested.

[c] — = no growth.

Source: Adapted with permission from C.J. Moir and M.J. Eyles, Inhibition, Injury and Inactivation of Four Psychrotrophic Foodborne Bacteria by the Preservatives Methyl p-Hydroxybenzoate and Potassium Sorbate, *Journal of Food Protection*, Vol. 55, pp. 360–366, © 1992, International Association of Milk, Food and Environmental Sanitarians, Inc.

ditions including log-phase ATR, a pH-dependent, stationary-phase ATR, and a pH-independent, general stress resistance induced in the stationary phase (AR).[43,67] Cells grown to stationary phase in minimal medium were 1,000-fold more resistant to acid pH than log-phase cells, 1% survived suspension in minimal medium at pH 3.0 for 60 minutes compared with 0.001% of log-phase cells.[67] Stationary-phase cultures with a final pH of 4.3 showed 1% survival after 4 hours at pH 3.0, compared with a survival rate of 0.001% for stationary-phase cultures with a final pH of 7.3.[67] The log-phase ATR appeared to involve two stages: (1) synthesis of a pH homeostasis system, ie, inducible amino acid decarboxylases, and (2) formation of about 50 acid-shock proteins, believed to prevent damage or repair damaged macromolecules. Three regulatory proteins (RpoS, Fur, and PhoP) have been described, each of which controls expression of a distinct set of acid-shock proteins. In a comparison of *S. typhimurium, E. coli,* and *Shigella flexneri* grown in minimal medium, *S. typhimurium* UK1 (a virulent strain) showed stationary-phase ATR that resulted in a 500-fold increase in survival after exposure to pH 3.0 for 4 hours, *S. flexneri* failed to show significant stationary-phase ATR at pH 3.0 while both unadapted and adapted *E. coli* (a toxigenic strain) showed acid resistance[73] (Figure 8-6). *S. typhimurium* UK1 grown to stationary phase in minimal medium also showed a general stress reaction that resulted in acid resistance at pH 3.0 but, in contrast to *E. coli* and *S. flexneri,* after growth in complex or minimal medium did not show acid resistance when exposed to pH 2.5.[73]

Some enterohemorrhagic (EHEC) strains of *E. coli* and some strains of *S. flexneri* grown overnight in complex medium were reported to show acid tolerance (Table 8-11).[5] In the most highly acid-tolerant EHEC strain, this tolerance was greatest in late stationary-phase cells. The acid tolerance was reported not to result from acidification of the overnight culture, the pH of which remained higher than 7.3.

In a study of a range of laboratory and commensal strains of *E. coli* and EHEC strains of O157:H7 the cells were grown in medium (LBG) containing (g l^{-1}) tryptone, 10; yeast extract, 5; NaCl, 5; glucose, 4; the final pH after growth being about 5.[74] After overnight growth, the cells were diluted in LBG medium adjusted to pH 2.0 with HCl and incubated at 37°C. After incubation for 6 hours the percent survival for two laboratory strains was 40% and <0.01%, respectively. The percent survival for three commensal strains was between 47% and 90% and that for 11 EHEC strains was between 6% and 81%. Growth and/or testing in minimal medium resulted in much lower survival, which could be increased by the addition of glutamate or arginine. Evidence was presented that resistance to acid pH could be maintained during storage at 4°C for up to 28 days.

Buchanan and Edelson[15] showed that growth of seven EHEC strains in tryptic soy broth (TSB) supplemented with 10 g glucose l^{-1} for 18 hours at 37°C ensured that the cultures reached a final pH

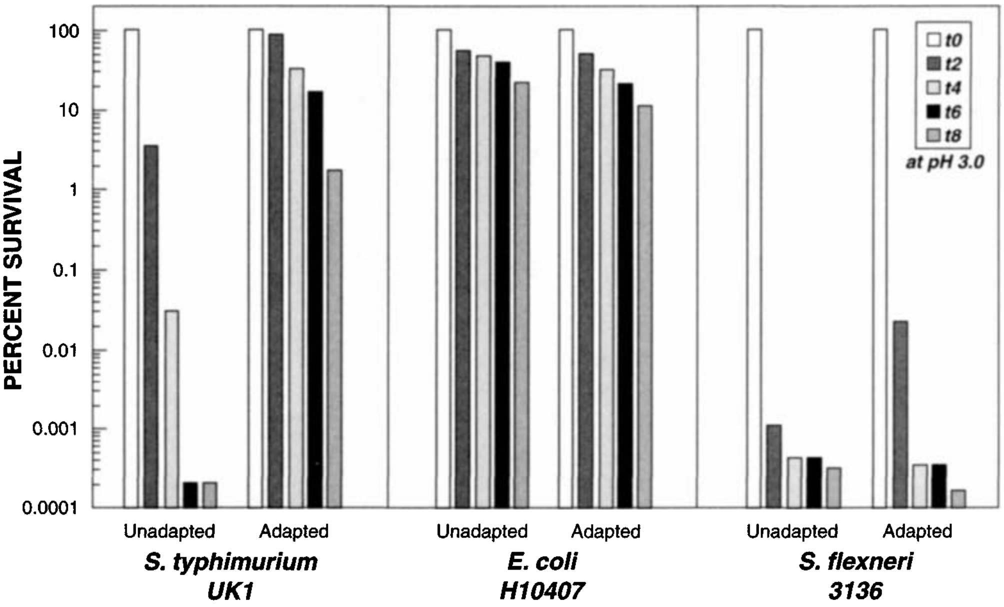

Figure 8-6 Stationary-Phase Acid Tolerance Responses in *Salmonella typhimurium, Escherichia coli,* and *Shigella flexneri.* Cells were grown overnight in minimal glucose medium (pH 7.7), harvested and resuspended to 2 × 10^8 cells ml^{-1} directly at pH 3.0 (unadapted) or to pH 4.3 (adapted) for 2 hours prior to resuspension at pH 3.0. Medium was adjusted to pH 3.0 or 4.3 with HCl. Bars represent average % survival (± 0.5 times the stated value) at 0 to 8 hours after acid challenge. *Source:* Reprinted with permission from J. Lin et al., Comparative Analysis of Extreme Acid Survival in *Salmonella Typhimurium, Shigella Flexneri,* and *Escherichia Coli, Journal of Bacteriology,* Vol. 177, pp. 4097–4104, © 1995, American Society for Microbiology.

between 4.6 and 5.2, resulting in maximum acid resistance. Control cultures in TSB without glucose reached a final pH of 6.9–7.0. Subsequent exposure of the cells to brain heart infusion medium adjusted to pH 2.5 and to pH 3.0 with HCl showed that all seven EHEC strains were acid resistant after growth in TSB plus glucose, ie, at pH 2.5 and 37°C they showed less than a 1.0 log decrease in viable count after 7 hours (Table 8-12). After growth in TSB without glucose there was a considerable difference in the acid tolerance of different strains, ie, at pH 2.5 the log decrease in viable count after 7 hours ranged from <0.3 to >3.8. Four of the EHEC strains were relatively acid sensitive when grown in TSB minus glucose, ie, these strains showed pH-dependent, stationary-phase acid resistance, whereas three of the EHEC strains were acid resistant after growth in TSB with or without glucose, ie, they showed pH-independent, stationary-phase acid resistance. Comparison of the counts on a nonselective medium and on a selective medium indicated that many of the surviving bacteria were sublethally injured and that the proportion of sublethally injured cells was typically greater in glucose-grown cells than in those grown without added glucose. This suggested that resistance to moderately acidic conditions allowed injured cells to remain viable for a longer time.

Exponential-phase cells of *L. monocytogenes* F4642 (Scott A) growing in brain heart infusion medium showed an acid tolerance response that was probably dependent, at least in part, on protein synthesis (Figure 8-7).[32] The ATR was induced optimally between pH 4.8 and 5.2. This bacterium also developed increased acid resistance upon entering the stationary phase; this resistance appeared to be independent of the pH-dependent, exponential-phase acid tolerance and to confer a much greater acid resistance[32] (Figure 8-8).

Response to other stress conditions can increase the acid tolerance of bacteria, for example, heat shock of seven strains of *E. coli* O157 at 48°C for 10 minutes increased the percent of survivors after incubation in a minimal medium at pH 2.5 and 37°C for 6 hours by factors of between 6.5- and 136-fold.[112]

Increased resistance to acid pH can also result in increased resistance to weak organic acids. For example, acid habituation of *E. coli* at pH 5.0 increased survival at pH 3.5 in the presence of lactic acid (31 mM), and acetic, propionic, sorbic, and benzoic acids (12.5 mM).[51] Exponential-phase ATR reduced the sensitivity of *S. typhimurium* to acetic, butyric, and propionic acids at pH 4.4, and stationary-phase ATR reduced the sensitivity to acetic and propionic acids (150 mM acetate or 100 mM propionate) but not to

Table 8-11 Acid Tolerance of Strains of Enterohemorrhagic *Escherichia coli* and *Shigella flexneri*. Fresh overnight cultures in Luria broth (LB) pH 7.3 were diluted 1:1,000 in LB adjusted to pH 2.5 and 3.0 with HCl and incubated at 37°C for 2 hours.

		Survival (%)	
Strain	*Serotype*	*pH 2.5*	*pH 3.0*
E. coli			
AD305	0157:H7	100	100
AD314	0157:H7	0.1	28
AD316	0157:H7	10	26
AD317	0157:H7	72	80
AD318	0157:H7	17	32
AD319	0157:H7	0.65	44
AD320	026:H	16	53
AD306	026:H30	3	36
AD307	026:H11	26	74
AD308	0111:NM	0.43	58
AD313	0111:NM	0.38	35
AD309	022:H8	6	38
AD310	015:H27	20	40
AD312	0165:H25	62	76
S. flexneri			
AD321	5	98	111
AD322	5	92	79
AD323	2a	12	10
AD324	5	3	10

Source: Reprinted with permission from M.M. Benjamin and A.R. Datta, Acid Tolerance of Enterohemorrhagic *Escherichia Coli*, *Applied and Environmental Microbiology*, Vol. 61, pp. 1669–1672, © 1995, American Society for Microbiology.

Table 8-12 Acid Resistance of Enterohemorrhagic *Escherichia coli*. Cultures were grown at 37°C for 18 hours in tryptic soy broth (TSB) plus glucose (when the final pH was 4.6–5.2) or in TSB minus glucose (when the final pH was 6.9–7.0). The bacteria were resuspended in brain heart infusion broth adjusted to pH 2.5 with HCl and incubated at 37°C for up to 7 hours. Viability was assessed by plating on brain heart infusion agar.

	% Survival after 7 Hours at pH 2.5	
E. coli *Strain and Serotype*	*Cells Grown in TSB Plus Glucose*	*Cells Grown in TSB Minus Glucose*
B 1409, 0157:H7	71	1
45753-35, 0157:H7	33	6
30-2C4, 0157:H7	98	62
932, 0157:H7	52	0.016
Ent-C9490, 0157:H7	42	45
A9124, 0157:H7	54	0.018
95JB1, 0111:H^{-}	79	54
ATCC 25922[a]	3.7	0.0002

[a] Biotype 1 reference strain, serotype unavailable.

Source: Calculated using data from R.L. Buchanan and S.G. Edelson, Culturing Enterohemorrhagic *Escherichia Coli* in the Presence and Absence of Glucose as a Simple Means of Evaluating Acid Tolerance of Stationary-Phase Cells, *Applied and Environmental Microbiology*, Vol. 62, pp. 4009–4013, © 1996, American Society for Microbiology.

benzoic acid (10 mM benzoate).[2] Conversely, exposure of *E. coli* grown in complex medium at pH 6.5 to 0.1% butyrate or propionate for 1 hour before incubation in medium adjusted to pH 3.5 with HCl for 30 minutes resulted in an increase in survival of between 50- and 200-fold compared with that of cells grown at pH 6.5 without exposure to the weak acid.[54]

A strain of *E. coli* O157 multiplied in a complex medium at pH 7 incubated at 10°C, but not in the medium acidified to pH values between 4.0 and 5.2 with acetic, citric, lactic, malic, mandelic, or tartaric acid (between 1 and 8 mM).[25] At 10°C inactivation occurred at greater rates in the presence of citric, malic, or mandelic acid than in the presence of acetic or lactic acid. At 4°C and pH values between 4.0 and 5.2, survival for periods of 7 to 56 days was greater in the presence of organic acids (eg, acetic acid, 27 mM) than in medium at pH 7.0 without added organic acids.[25]

Survival of stationary-phase *L. monocytogenes* at low pH was reduced by the presence of acetic or lactic acid.[47] Total acetic acid concentrations of 5,000–10,000 mg l^{-1} at pH 3.5, equivalent to undissociated acetic acid concentrations of 79–158 mM, caused a considerable increase in the death rate at 20°C; 10,000 mg total acetic acid l^{-1} causing a decrease by about 10^4 in the number of viable bacteria in four days (Figure 8-9).

Acetic acid has been used as a means of inactivation of Enterobacteriaceae, in particular *Salmonella* spp., in sauces, the criterion being three decimal reductions of *Escherichia coli* in 72 hours at 20°C for sauces with a pH between 3.0 and 4.5 to give a product that is intrinsically safe.[24] The concentrations of salt and sugar present would also influence the death rate. Sauces of this kind are liable to taste strongly acidic or salty and now tend to have been superceded by milder, less acidic products containing additional preservatives (see Chapter 30).

It is evident that the prehistory of microorganisms, including the growth conditions, and the conditions of exposure to acid pH can have a profound effect on survival at low pH. This must be taken into account in any consideration of the ability of pathogenic bacteria and other microorganisms to survive in foods. Methods such as that described by Buchanan and Edelson,[15] to ensure that bacteria show maximum acid resistance, need to be used in experimental work designed to assess the ability of bacteria to survive in acidic

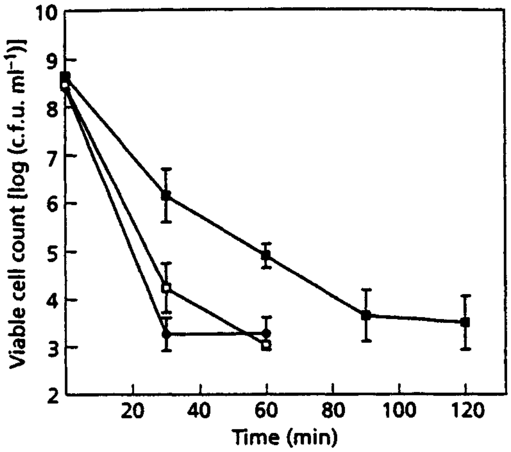

Figure 8-7 The Acid Tolerance Response of *Listeria monocytogenes* Scott A. Cultures were grown to exponential phase (~ 10^8 cfu ml^{-1}) and either preadapted for 1 hour at pH 5.8, ■; left untreated, ●; or preadapted in the presence of 100 μg chloramphenicol ml^{-1}, ☐. (The chloramphenicol was added 15 minutes prior to reducing the pH to 5.8.) The pH of each culture was then reduced to pH 3.0 with HCl and cell viability measured at the time intervals shown. *Source:* Reprinted with permission from M.J. Davis, P.J. Coote, and C.P. O'Byrne, Acid Tolerance in *Listeria Monocytogenes:* The Adaptive Acid Tolerance Response (ATR) and Growth-Phase-Dependent Acid Resistance, *Microbiology,* Vol. 142, pp. 2975–2982, © 1996, Society for General Microbiology.

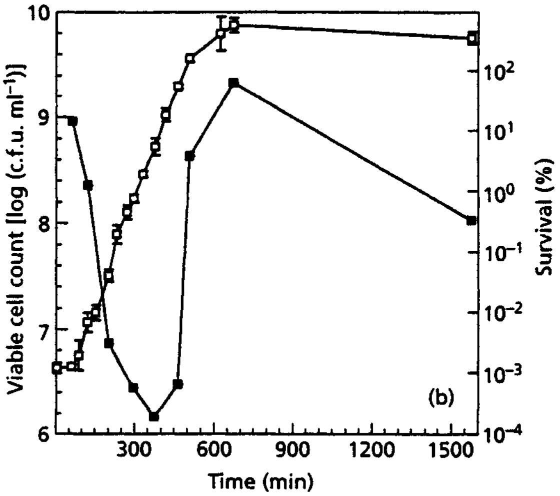

Figure 8-8 Growth-Phase–Dependent Acid Tolerance in *Listeria monocytogenes* Scott A. Acid resistance was measured throughout growth. Growth was followed by viable cell counts, ☐; and samples were removed throughout growth to test acid resistance, ■. Viable cell counts were made immediately before and after an acid challenge at pH 3.0 for 90 minutes and results are shown as % survival. *Source:* Reprinted with permission from M.J. Davis, P.J. Coote, and C.P. O'Byrne, Acid Tolerance in *Listeria Monocytogenes:* The Adaptive Acid Tolerance Response (ATR) and Growth-Phase-Dependent Acid Resistance, *Microbiology,* Vol. 142, pp. 2975–2982, © 1996, Society for General Microbiology.

foods. Acid-adapted *E. coli* O157:H7 (grown in medium adjusted to pH 5.0 with HCl) survived better than nonadapted cells in buffer adjusted to pH 3.85 with 125 mM lactic acid, and also survived better during sausage fermentation by lactobacilli, in hard salami, and in apple cider at pH 3.46.[69] After growth in sterilized orange juice at pH 5.0, four species of *Salmonella* survived at 4°C without a decrease in numbers for 0.21 days, 2.44 days, 4.23 days, and 11.02 days in orange juice adjusted to pH 3.5, 3.8, 4.1, and 4.4, respectively, with citric acid.[88] In addition to the above factors, the possibility of previous exposure of microorganisms to subinhibitory concentrations of weak organic acids should be considered in assessing the preservative effect of these acids in foods.

8.4 MECHANISM OF EFFECT OF pH, ACIDULANTS, AND ORGANIC ACID PRESERVATIVES

Acidulants exert their antimicrobial effect in foods partly by reducing the pH of the food in contact with microorganisms and partly due to the specific properties of the acid. In studies of the mechanism of action of antimicrobials the most significant effects are those that occur at concentrations similar to those that reduce the rate of growth and approach the MICs. Sorbic acid has been reported to affect cell morphology and hydrophobicity and to inhibit spore germination.[101] Both sorbic and benzoic acids have been shown to inhibit a range of enzymes.[21,101] In order to understand the extent to which these effects are the primary cause of inhibition or lethality, it is necessary to consider the relative concentrations necessary for these effects. Studies of bacteria and of yeasts have indicated that uptake of weak organic acids and the influence of the protons and anions within the cytoplasm are a primary cause of inhibition.[33,100]

The permeability of microbial cell membranes to protons is low, so that passive influx of protons from the external medium into the microbial cell is probably limited. The changes measured in the internal pH (pH_i) of microorganisms caused by changes in external pH (pH_o) are generally relatively small, and the cytoplasm tends to be maintained at a pH near to neutrality.[9,56] Maintenance of the internal pH results

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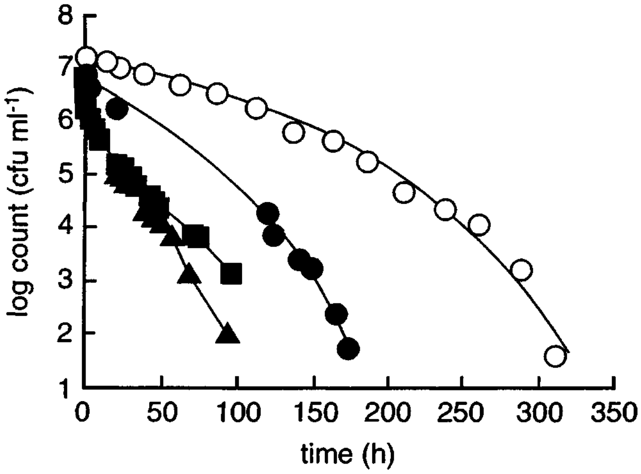

Figure 8-9 The Effect of Total Acetic Acid on Survival of *Listeria monocytogenes* F6861 at pH 3.5 and 20°C. Culture medium as in Figure 8-4 (A). ○, no added acetic acid; ●, 5,000 mg acetic acid l^{-1}; ■, 7,500 mg acetic acid l^{-1}; ▲, 10,000 mg acetic acid l^{-1}. *Source:* S.M. George, L.C.C. Richardson, and M.W. Peck, unpublished results UK Crown Copyright 1997. These data were produced as part of the MAFF Predictive Microbiology Programme that led to the production of the software package Food Micromodel. This package is available from Leatherhead Food RA, Leatherhead, Surrey, UK.

partly from the very low permeability of the cell membrane to protons and partly from the buffering capacity of the cytoplasm. Active pH homeostatis in neutrophilic and acidophilic bacteria depends on expulsion of protons through the cell membrane and a corresponding movement of potassium ions into the cell.[56] Extreme lowering of the external pH is liable to cause some decrease in internal pH; eventually the difference between the internal and external pH, Δ pH, reaches a maximum, Δ pH begins to collapse, and the cells begin to die.

The undissociated form of lipophilic acids, due to lack of charge and to hydrophobicity, is able to diffuse through the cell membrane of microorganisms,[55,116] and on entering the cell, where the pH is usually higher than that of the external environment, some of the acid dissociates tending to cause accumulation of protons and preservative anions in the cell cytoplasm. An investigation of the effect of propionic, benzoic, and sorbic acids on *E. coli* indicated that their inhibitory effect was due predominantly to the undissociated acid.[100] There was evidence that inhibition of growth resulted from two effects: (1) a specific inhibition of an unidentified metabolic function by the accumulation of undissociated acid, and (2) a generalized inhibition caused by acidification of the cytoplasm. In the case of propionic, benzoic, and sorbic acids, the former, specific effect was greater. The fact that the molar concentration of sorbic acid required to inhibit *Saccharomyces cerevisiae* was 1/30 that of acetic acid, with a similar pK_a, is further evidence of a specific effect of these acids.[107]

The mechanism for export of metabolically produced organic acids has been studied particularly in lactic acid bacteria.[1] *Lactobacillus helveticus* produces large quantities of lactic acid, which can lower the pH of the external medium to about 3.5; this bacterium has a carrier-mediated system that enables export of undissociated lactic acid or of the lactate anion in symport with one proton.[44]

It has been estimated that MICs for undissociated forms of these weak acids are between 10-fold and 600-fold greater than those of the dissociated forms (Table 8-13).

Strains of *Zygosaccharomyces bailii* are relatively resistant to preservatives and are a major cause of spoilage of acidic foods. Growth of *Z. bailii* and many other species of yeast in the presence of benzoate or sorbate induced increased resistance to these antimicrobials.[113–116] The cells were permeable to the undissociated form of these acids and evidence was reported that increased resistance to these preservatives in many yeasts resulted primarily from an inducible, energy-requiring system that trans-

Table 8-13 Experimentally Determined Values for MICs of Undissociated and Dissociated Organic Acids

Organism	*Acid*	*MIC_u*[a]	*MIC_d*[b]	*Reference*
Escherichia coli M23	lactic	8.32		95
Yersinia enterocolitica	lactic	5–10		12
E. coli	propionic	70	800	36
Staphylococcus aureus	propionic	19	830	36
Bacillus cereus	propionic	17	380	36
E. coli	sorbic	1	100	36
E. coli	sorbic	1	350	97
S. aureus	sorbic	0.6	400	36
B. cereus	sorbic	1.2	110	36
Listeria innocua	lactic (as sodium lactate)	*ca.* 0.8–5.0		57

[a] MIC_u, MIC of undissociated acid, mM.

[b] MIC_d, MIC of dissociated acid, mM.

Source: Reprinted with permission from K.A. Presser, D.A. Ratkowsky, and T. Ross, Modelling the Growth Rate of *Escherichia Coli* as a Function of pH and Lactic Acid Concentration, *Applied and Environmental Microbiology*, Vol. 63, pp. 2355–2360, © 1997, American Society for Microbiology.

ported the preservative anion out of the cell. Growth in the presence of low concentrations of preservative stimulated fermentation and resulted in reduced growth yields and reduced growth rates. This was attributable to the increased energy required to maintain the intracellular pH by exporting protons, and to transport the preservative anion out of the cell.[114–116,120] The cell membrane of *Z. bailii* had a relatively low permeability to benzoic acid, and growth in the presence of benzoic acid resulted in a further reduction in the permeability of the cell to benzoic acid and, to a lesser extent, to propionic acid.[118] For a range of 23 yeasts, a correlation was found between the MIC of propionic and benzoic acids and the reciprocal of rate of uptake and the reciprocal of the permeability of the cell membrane to propionic acid, indicating that resistance of yeasts to these acids was associated with relatively low permeability of the cell membrane to these compounds.[119] Growth of *Z. bailii* in the presence of glucose and low concentrations of benzoic acid resulted in stimulation of fermentation and only low concentrations of benzoate were accumulated in the cytoplasm.[120] At higher concentrations of benzoate, near to the MIC, fermentation was inhibited leading to inhibition of growth. This was correlated with an increase in the internal concentration of benzoate, and pH_i was lowered by less than one pH unit to about pH 6.4, remaining much higher than that of the external medium, pH 3.5.[120] A study of the effect of increasing concentrations of benzoic acid on glycolysis reactions suggested that limitation of ATP was an important factor in the inhibition. The presence of benzoic acid resulted in a similar response of *Saccharomyces cerevisiae*, concentrations near to the MIC resulted in a decrease in pH_i of about one pH unit to approximately pH 5.8, compared with pH 3.5 in the culture medium; in contrast to *Z. bailii*, the levels of hexosephosphates were lowered.[121]

Concentrations of sorbic acid that reduced the growth rate of *S. cerevisiae* caused little reduction of internal pH, which remained at between pH_i 5.4 and 5.8 (external pH 3.8), but caused increased consumption of ATP by the cells, reducing the energy available for growth and cell division.[10] This was attributable to increased activity of a membrane-bound H^+-ATPase that coupled hydrolysis of ATP to expulsion of protons from the cell. When grown in the presence of benzoic acid, *S. cerevisiae* was also able to extrude benzoic acid by an energy-dependent process that probably involved a membrane-bound ATPase.[55] Evidence was reported that growth of *S. cerevisiae* in the presence of sorbate, 1 mM, at pH 4.5 induced two plasma membrane proteins, one of which catalyzed energy-dependent extrusion of sorbate and benzoate,[90] demonstrating the involvement of the type of mechanism suggested by Warth[113] and Henriques et al.[55]

At concentrations approaching the MIC, depletion of ATP may be a major factor leading to inhibition of microorganisms. Consequent inactivity of membrane-bound ATPases could lead to an accumulation of protons and preservative anions in the cytoplasm; both of these factors will then influence many intracellular enzyme reactions.

Several types of mechanism have been described that contribute to inducible acid tolerance and acid resistance in microorganisms.[4,97] Lowering of the internal pH can induce amino acid decarboxylases in some species; the amino acid decarboxylation can elevate the internal pH. The transition of bacteria from the exponential phase to the stationary phase is correlated with an increase in acid resistance and in general resistance to stress. Entry into the stationary phase is accompanied by a modification of sigma factors, proteins whose primary role is to bind to the core RNA polymerase, conferring promotor specificity.[76] The starvation sigma factor, σ^S (RpoS), encoded by the *rpoS* gene, has been identified as controlling stationary-phase adaptive processes in *E. coli*. A primary role of σ^S is as a factor controlling the production of stress-response proteins, induced during transition from the exponential phase to the stationary phase, but σ^S also has a role in the production of exponential-phase proteins.[4,43,76] Other regulatory proteins are also involved in response to acid stress.[43] Evidence has also been reported that during acid habituation of *E. coli* monounsaturated fatty acids present in phospholipids were either converted to their cyclopropane derivatives or replaced by saturated fatty acids, and it was suggested that increased levels of cyclopropane fatty acids may enhance the survival of microorganisms exposed to low pH.[13] During growth of *Clostridium acetobutylicum*, a decrease in pH of the medium from 6 to 5 was associated with a decrease in the ratio of unsaturated to saturated fatty acids and an increase in cyclopropane fatty acids.[68]

8.5 FACTORS INFLUENCING THE EFFECT OF pH, ACIDULANTS, AND ORGANIC ACID PRESERVATIVES

Combinations of inhibitory factors may be used in many foods, these combinations of factors may have effects that are additive, synergistic, or antagonistic; methods of distinguishing were discussed by Parish and Davidson.[87] An additive effect is one in which the combined effect of two (or more) antimicrobials is equal to the sum of the effect of each antimicrobial acting individually, whereas a synergistic effect is one in which the combined effect is greater than the sum of the effect of each antimicrobial. Clearly, the combined antimicrobial effect of pH and a preservative such as sorbic acid can be synergistic. For example, Figure 8-1 shows that adjustment of the pH of the medium to 5.1 with HCl had no effect on the probability of growth from single vegetative bacteria of proteolytic *Clostridium botulinum* at 30°C in 3 days, and 1,000 mg total sorbic acid l^{-1} in a medium at pH 7.0 had no effect on the probability of growth of single vegetative bacteria of proteolytic *C. botulinum* at 30°C in 3 days,[79] but the combined effect of addition of 1,000 mg total sorbic acid l^{-1} and adjustment of the pH to 5.1 reduced the probability of growth in 3 days by a factor greater than 10^5-fold (Table 8-9).

The antimicrobial effect of pH, acidulants, and weak organic acid preservatives in foods will be influenced by many of the components of the food including available sugars and amino acids and by the temperature at which the foods are maintained. The water activity (a_w) of a food could affect the pK_a of weak acids, and thus alter their antimicrobial effect, although in water-glycerol mixtures with an a_w between 0.80 and 1.0, the pK_a of sorbic acid at 25°C only varied between approximately 4.62 and 4.80.[89] The effect of acidulants and preservatives will depend on their chemical stability in the food and on whether they are degraded by microorganisms present. In many cases, the acidulants, such as acetic and lactic acids, are likely to have an additive or possibly a synergistic influence on the effect of weak organic acid preservatives, such as benzoic and sorbic acids. Many reports have been published describing the effect of these factors, singly and in combination, in foods.

In addition, the effect of organic acid preservatives is liable to be influenced greatly by the presence of a lipid phase. Uptake of lipid-soluble antimicrobials, in particular sorbic and benzoic acids and hydroxybenzoates, occurs into the lipid phase, reducing the effective concentration in contact with microorganisms contained in the aqueous phase. Only the undissociated acid is soluble in the lipid phase.

The distribution coefficient of sorbic acid, for example, can be defined as:

$$\frac{\text{concentration of sorbic acid in the lipid phase}}{\text{concentration of undissociated sorbic acid in the aqueous phase}}$$

The distribution coefficient defined in this way is independent of pH value and enables calculation of the amounts of undissociated and dissociated sorbic acid in the aqueous phase at any pH.[77] The distribution coefficients in peanut oil:water at 20°C were reported as: sorbic acid, 3.1 (95% confidence limits ±0.13); ethyl hydroxybenzoate, 26.0 (95% confidence limits ±1.5); propyl hydroxybenzoate, 87.5 (95% confidence limits ±2.1); and benzoic acid, about 6 at very low concentrations and about 13 at very high concentrations.[77] The solubility of these preservatives is similar in most types of oil, thus the distribution coefficients in other types of oil are probably similar to those reported above. For sorbic acid in mixtures of oil and water at about pH 5.0, roughly the same concentration of undissociated sorbic acid would remain in the water phase irrespective of the amount of oil present in the system (Figure 8-10). As the pH is decreased below 5.08, any increase in the ratio of oil to water in the system would result in a decrease in the concentration of undissociated sorbic acid in the aqueous phase. A greater proportion of parabens is taken up into the oil phase (Figure 8-11).

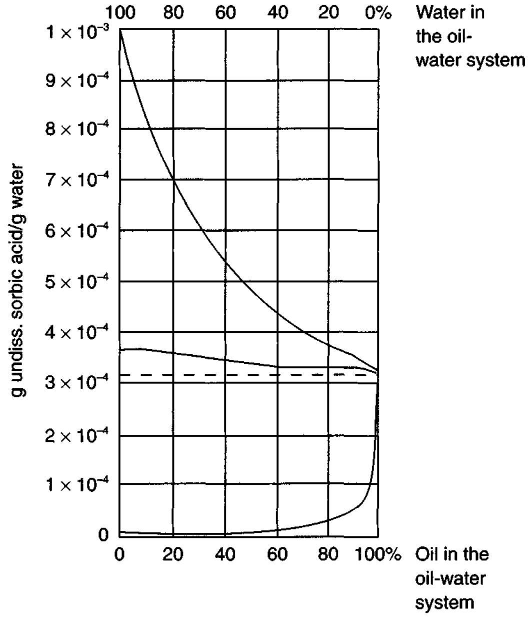

Figure 8-10 Concentration of Undissociated Sorbic Acid (g g^{-1} water) on Addition of 0.1 g Sorbic Acid to 100 g (water + peanut oil) at Various pH Values and with Various Amounts of Oil in the Oil-Water System. Temperature 20°C. Top curve, pH = 2.0; middle curve, pH = 5.0; dashed line, pH = 5.08; bottom curve, pH = 7.0. *Source:* Reprinted with permission from M. Lubieniecki-von Schelhorn, Investigations of the Distribution of Preservatives between Fat and Water in Foods, in *Microbial Inhibitors in Food,* N. Molin and A. Erichsen, eds., pp. 139–144, © 1964, Almqvist and Wiksell.

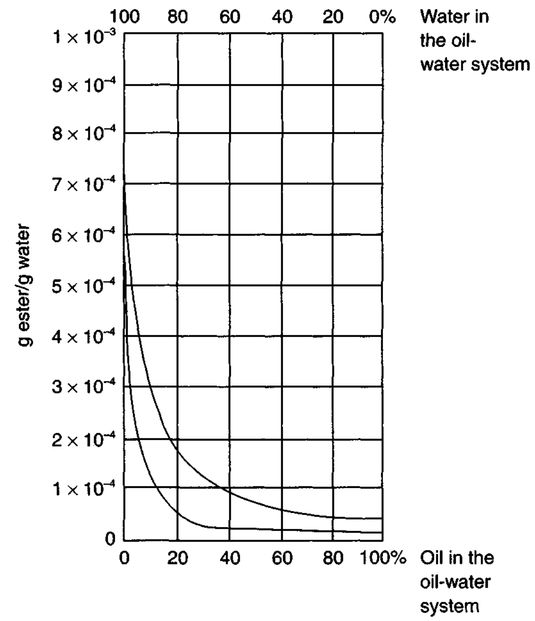

Figure 8-11 Concentration of Ethyl and Propyl Esters of p-Hydroxybenzoic Acid (g g^{-1} water) on addition of 0.1 g of an Ester to 100 g (water + peanut oil) with Various Amounts of Oil in the Water-Oil System. Temperature 20°C. Upper curve, ethyl ester; lower curve, propyl ester. *Source:* Reprinted with permission from M. Lubieniecki-von Schelhorn, Investigations of the Distribution of Preservatives between Fat and Water in Foods, in *Microbial Inhibitors in Food,* N. Molin and A. Erichsen, eds., pp. 139–144, © 1964, Almqvist and Wiksell.

At the concentrations used in foods organic acids have little effect on the distribution of sorbic acid between oil and water phases, but the presence of high concentrations of glucose, sucrose, or sodium chloride reduces the solubility of sorbic acid in water.[49] It is clear that the antimicrobial effect of these preservatives is liable to be reduced markedly by the presence of fat in foods, eg, in emulsions, that the reduction in effect will be greatest in acidic foods, and that the reduction in activity will be greater in the case of hydroxybenzoates and benzoic acid than in the case of sorbic acid.

In foods that contain solid components, preservative factors may be less effective than in liquid foods because attachment of microorganisms to surfaces can increase their resistance to acid pH[93] and to other inhibitory factors. Immobilization of *Acetobacter aceti* in alginate beads or, to a lesser extent, adsorption on cellulose beads were reported to protect the bacterium from the effect of acetic acid,[65] although apparently the pH of these experiments was not reported.

8.6 CONCLUDING REMARKS

Microorganisms differ widely in their ability to multiply and to survive at low pH and in the presence of weak organic acids. An understanding of the effect of pH, acidulants, and organic acid preservatives on ability of microorganisms to survive and multiply is important as a basis for the most effective utilization of these factors in preservation of foods and in the elimination of microorganisms. Many reports have been published describing the effect of these factors, singly and in combination, on microorganisms in foods, and predictive models of the combined effect of temperature, pH, and acetic or lactic acid on growth of *Listeria monocytogenes* and of *E. coli* have been developed.[46,94] A great deal of information is available regarding the mechanisms of inhibition by pH and weak acids and of the mechanisms of microbial adaptation to multiply or to survive in the presence of these antimicrobial factors but further work is needed to provide an integrated understanding of these mechanisms.

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9 The Use of Other Chemical Preservatives: Sulfite and Nitrite

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9.1 INTRODUCTION

There is a widely recognized consumer-led desire to reduce the use of chemical additives, including antimicrobial preservatives, in foods.[31] However, many chemical preservatives continue to play an important role in ensuring satisfactory shelf life and safety of a wide range of foodstuffs. Antimicrobial preservatives used in foods, summarized in Table 2-6, Chapter 2, include acidulants (eg, inorganic acids such as hydrochloric and phosphoric; organic acids such as acetic, citric, lactic, malic, and tartaric), weak, lipophilic organic acid preservatives (eg, propionic, sorbic, benzoic acids, and esters), weak inorganic acids (sulfite, nitrite, carbon dioxide in modified atmosphere packaging), antibiotics (eg, the bacteriocin nisin, and the antimycotic natamycin [pimaricin]) and other products of lactic acid bacteria. Acidulants, organic acids, and esters are discussed in Chapters 8 and 31. The use of carbon dioxide in modified atmosphere packaging is covered in Chapter 10. Chapter 12 deals with nisin and other culture products, and Chapter 13 covers the other antimicrobials that occur naturally in foods. The most important of the other antimicrobial preservatives, the weak inorganic acids sulfite and nitrite, are covered in this chapter.

9.2 SULFITE

9.2.1 General

Sulfur, as an element, was well known in ancient times when the burning of sulfur was valuable as a means of purification and, as we say now, disinfection.[73] The burning of sulfur in containers, or in areas where foods and drinks were to be stored or prepared, generated sulfur dioxide gas (SO_2), providing a link with modern practice because sulfur dioxide is the predominant form in which sulfite is taken into microbial cells and inactivates them. However, today, the compound most often used to generate sulfur dioxide and the various anions with which it comes into equilibrium in foods is sodium metabisulfite ($Na_2S_2O_5$). Other compounds frequently used as sulfiting agents include sulfur dioxide itself, sodium and potassium salts of sulfite (Na_2SO_3; K_2SO_3), bisulfite ($NaHSO_3$; $KHSO_3$), and potassium metabisulfite ($K_2S_2O_5$). Calcium salts are also sometimes used. These salts all share the ability to generate free molecular SO_2, which correlates extremely well with their preservative activity. For this reason it is usual to calculate treatment levels for the various salts on the basis of their "SO_2 equivalents."

9.2.2 Uses in Foods

Sulfur dioxide is very reactive chemically[93] so it is not surprising that it is unusually multifunctional as a food additive. Its uses include: (1) the prevention of oxidative rancidity and other oxidation reactions, (2) the prevention of enzymic and nonenzymic browning reactions, (3) a bleaching agent and a color stabilizer, (4) the stabilization of ascorbic acid (vitamin C), (5) a flour improvement agent, and (6) an antibacterial and antimycotic (Table 9-1).[32,55,65,93]

The major uses of sulfur dioxide are in fruit and vegetable products and in alcoholic and nonalcoholic drinks, as well as a few applications in raw meat products and fish. In fruits and vegetables, sulfite is used mainly to prevent chemical and enzymic reactions that lead to deterioration, eg, in dried products or in products in which the water activity (a_w) has been otherwise reduced by the addition of solutes. Sulfite is used in drinks where the pH is low enough for an antimicrobial effect, mainly to prevent the growth of yeasts and molds. Applications in raw meat products aim to slow the growth of microorganisms and extend shelf life. An additional role of sulfite, not typical of other applications, is the improvement of safety through inhibition of the growth of Gram-negative food poisoning bacteria.[7,8] Since the functions of sulfite in foods vary widely, the applications and the concentrations required to perform a particular function vary widely as well. This is illustrated by the examples of sulfite usage worldwide (Table 9-2). The concentrations listed are illustrative because the maximum allowed levels differ in different countries.[61] In Europe, for instance, levels are authorized by European Union (EU) directives for sulfur dioxide (E220), sodium sulfite (E221), sodium bisulfite (E222), sodium metabisulfite (E223), potassium metabisulfite (E224), calcium sulfite (E226), calcium bisulfite (E227), and potassium acid sulfite (potassium bisulfite; E228).[61] In the United States (US), the use of sulfite in its various forms was generally regarded as safe (GRAS). However, reports that sulfite, particularly if inhaled as SO_2, could affect some individuals suffering from asthma has led to a more restricted and careful use.[54] The problem most often occurred following the use of high levels of sulfite as "salad fresheners" or by the addition of sulfite to acid foods, such as salads to which dressings are applied. Regarding the toxicity of ingested sulfite, in Europe the current acceptable daily intake for humans is 0.7 mg per kg body weight.[3] The average daily per capita consumption of sulfite from foodstuffs in Europe is about 20 mg SO_2, but may be five times more than this if alcoholic drinks are included.[2] The liver is capable of detoxifying gram quantities per day via the action of sulfite oxidase.[3] The efficacy of sulfite as an antimicrobial preservative (though not necessarily so in its other roles) is very pH dependent, for reasons that are well known (Section 9.2.3), with its effectiveness increasing greatly with reduction of pH. For this reason, products such as drinks, syrups, and purees are often acidified to ensure the required degree of preservation. This is particularly important in products such as syrups that contain high levels of sugars, because these products react chemically with sulfite and, because the reaction products are not antimicrobial, lead to a reduction in effectiveness during storage. The inhibitory activity of sulfite may fall during the storage of foods for other reasons also. For instance, in nonhermetically sealed containers it may oxidize to form sulfate, which is quite inactive in any of the functions for which sulfite is used. Many other reactions of sulfite with food ingredients lead to reductions in levels of free SO_2 (Section 9.2.3) so that, in contrast to most other food preservatives, it is important that such losses are taken into account, within legal constraints, in the design stages of product formulation, processing, and intended storage life.

Table 9-1 Preservative Functions of Sulfite in Foods

Function	*Example*
Antioxidant	Delays onset of oxidative rancidity and other oxidative deterioration reactions in foods during storage. Reduces color loss in meat, fruits, and vegetables. Aids retention of ascorbic acid (vitamin C) and carotenes during storage of foods.
Enzyme inhibitor	Prevents enzymic browning of fruits and vegetables caused by the activity of polyphenol oxidases.
Maillard reaction inhibitor	Prevents nonenzymic browning reactions between amino groups and reducing sugars by forming stable compounds with reaction intermediates.
Reducing agent	Improves rheology of flour by reacting with protein sulfydryl groups.
Antimicrobial	Inhibits growth of yeasts and molds in low pH and low a_w foods. Inhibits growth of Enterobacteriaceae and other Gram-negative bacteria in higher pH, high a_w foods.

Source: Reprinted from G.W. Gould and N.J. Russell, Sulphite, in *Food Preservatives*, N.J. Russell and G.W. Gould, eds., pp. 72–88, © 1991, Aspen Publishers, Inc.

Table 9-2 Major Applications of Sulfite in Foods and Levels of Use

Foods†	*Typical Use Concentrations (as SO_2: mg kg^{-1})*	*Major Purpose*‡
Fresh vegetables (onion, garlic, horseradish pulp)	50–1000	c, m
Frozen vegetables (white vegetables, mushrooms)	50	c
Dried vegetables	250–2500	c, o
Canned vegetables	20–200	c
Pickles	20–100	c, o
Peeled potatoes	10–50	c
Potato powders and flakes	100–500	c
Frozen potatoes	100	c
Fresh fruits	100	c, m
Dried fruits	100–2000	c, o
Fruit pulps, purees, and fillings	50–500	c, m
Fruit juices	10–100	c, m
Jams and jellies	50–100	c, m
Fruit-based sauces and related products	50–100	m
Sugar confectionary	50	c, m
Nonalcoholic beverages	20–200	m
Alcoholic beverages		
beers	10–30	m
wines	100–300	m
Vinegar	50–200	m
Sausage meat, burgers, and other meat products	450	c, m
Dried or salted fish	350–1000	c, o
Raw and frozen crustaceans	30–350	c, o

†Food uses and allowed concentrations vary greatly in different countries.
‡c: color retention; o: antioxidation; m: antimicrobial.

Source: Adapted from G.W. Gould and N.J. Russell, Sulphite, in *Food Preservatives*, N.J. Russell and G.W. Gould, eds., pp. 72–88, © 1991, Aspen Publishers, Inc.

9.2.3 Chemistry of Sulfur Dioxide

The simple oxyanions of sulfur in its tetravalent states are salts of sulfurous acid (H_2SO_3), which one would expect to be formed when sulfur dioxide dissolves in water. However, comparison of Raman and infrared spectra of dissolved SO_2 with gaseous or liquid SO_2 have indicated that very little H_2SO_3 exists in solution. Dissolved SO_2 exists predominantly as the hydrate $SO_2{\cdot}H_2O$, or as a clathrate inside a shell of about seven water molecules.

The important equilibria that become established in foods are to form bisulfite:

$$SO_2{\cdot}H_2O \rightleftharpoons HSO_3^- + H^+$$

The bisulfite can then dissociate to form sulfite:

$$HSO_3^- \rightleftharpoons SO_3^{2-} + H^+$$

The first dissociation constant has a p*K* value of 1.86 at 25°C and zero ionic strength while the second, under the same conditions, leading to the formation of the sulfite anion, has a value of 7.18.[93] Consequently, in acid foods, there will generally be a mixture of hydrated sulfur dioxide and bisulfite anion present, while in near-neutral pH foods, bisulfite and sulfite will predominate. Figure 9-1 shows the expected relative concentrations of the various forms as a function of pH.

A further equilibrium that is of relevance in foods is the condensation of two bisulfite anions to form metabisulfite:

$$2HSO_3^- \rightleftharpoons S_2O_5^{2-} + H_2O$$

This equilibrium is only significant at high concentrations of bisulfite so that, at the low concentrations of metabisulfite that are employed in food preservation, the equilibrium will be well to the left and metabisulfite added as a preservative will rapidly dissociate to give bisulfite, and $SO_2{\cdot}H_2O$, depending on the pH.

The chemical reactivity of sulfite results from its strong reducing activity and its ability to take part in nucleophilic attack. Table 9-3 lists some of the important reactions in a food context that sulfite can undergo. The reactions that are most important in its antimicrobial activity include those with carbonyl groups, because these occur in aldehydes and ketones, which are present in many critical biological molecules, including proteins and some enzyme cofactors (eg, pyridoxal phosphate, folic acid). Pyridoxine reacts with sulfite to form an addition compound, so that nicotinamide adenine nucleotide (NAD$^+$) and (NADP$^+$) reactions can be interferred with. Thiamine is cleaved by sulfite to form pyrimidine sulfonate and a thiazole-containing product.

Proteins that depend on disulfide bonds for maintenance of their tertiary structures are sulfite sensitive, because sulfite can rupture these bonds to form thiosulfonate and thiol pairs:

$$-RSSR- + SO_3^{2-} \rightleftharpoons -RSSO_3^- = -RS^-$$

In this way sulfite may inhibit many of the critical enzymes within the microbial cell. Likewise, oxidized glutathione (GSSG) is susceptible to disrup-

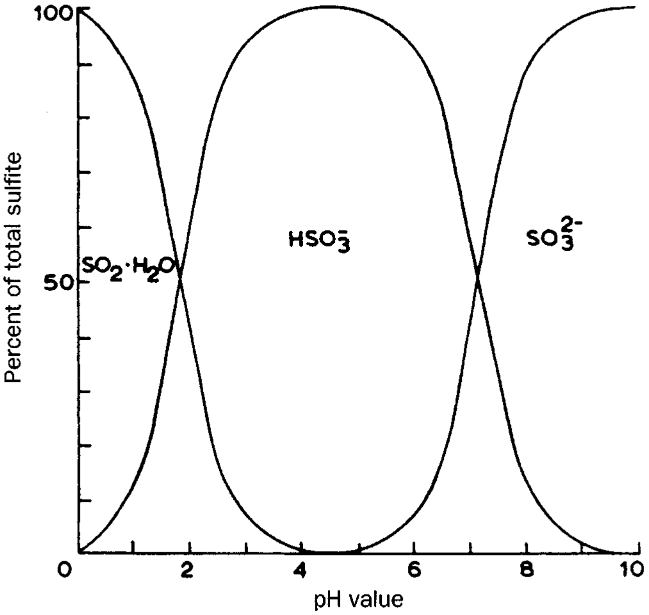

Figure 9-1 Relative Amounts of Sulfur Dioxide, Bisulfite, and Sulfite As a Function of pH. Plotted from the data of King et al.[43] *Source:* Copyright © 1989, A.H. Rose and B.J. Pilkington.

tion by sulfite. The pyrimidine bases cytosine and thymine in DNA and cytosine and uracil in RNA are subject to nucleophilic attack by sulfite. The purine adenine, present in adenine nucleotides (ATP; ADP; AMP), as well as in DNA and RNA, forms addition compounds with sulfite. Sulfite may accelerate the autoxidation of polyunsaturated fatty acyl residues in the lipids of yeasts and other fungi (but not bacteria).

There are therefore many means by which sulfite can interfere with the functioning of the most important molecules and reactions in microbial cells once it has gained access. The mechanism by which such access is gained is fundamental to the mode of action of sulfite as an antimicrobial preservative.

9.2.4 Antimicrobial Activity of Sulfite

The molecular mechanisms by which sulfite inhibits growth or inactivates microorganisms have received little attention, to some extent because it may react with so many critical components of a cell, as indicated above (Table 9-4). It is unlikely that there is just one specific reaction that is predominant in its effects on diverse organisms. The situation is made more complex by the effect of pH on the molecular form in which sulfite interacts with a microorganism. In different foods, the pH can range from below 3 to near neutrality. However, within the cytoplasm of bacteria, yeasts, and molds, homeostatic mechanisms operate to maintain a near-constant internal pH that is commonly near to 6.5.

Based on a close correlation observed between the intracellular accumulation of radioactivity from $^{35}SO_2$ in solutions at different pH values, and the estimated proportion existing as SO_2, it was proposed that it was molecular SO_2 that was taken up by cells of the yeast *Saccharomyces cerevisiae* var. *ellipsoideus*. While it was originally thought that active transport may have been involved, it was later demonstrated that SO_2 uptake by *S. cerevisiae* was predominantly passive, though a slow transport system for bisulfite ions that operated at low concentrations of the anion was tentatively suggested.[80]

S. cerevisiae accumulates SO_2 from solutions very quickly, reaching equilibrium within about 5 minutes and, at this time, the intracellular concentra-

Table 9-3 Major Food-Related Reactions Undergone by Sulfite

Sulfite-Reactive Chemical Group	*Examples of Biological Sources*
Carbonyl	Aldehydes, ketones
Disulfide bonds	Proteins, glutathione
Schiff's bases	Enzymes, cofactors
Pyridines	NAD^+, $NADP^+$
Pyrimidines	DNA, RNA
Quinones	Electron transport intermediates
Carbon-carbon double bonds	Fatty acids, enzyme cofactors

Source: Data from B.L. Wedzicha, *Chemistry of Sulphur Dioxide*, © 1984, Elsevier Applied Science.

Table 9-4 Sulfite-Sensitive Metabolic Systems in Microorganisms

Metabolic System	*Components Affected*
Intermediary metabolism	Enzymes, cofactors, prosthetic groups
Energy production	Enzymes, cofactors, electron carriers
Protein synthesis	Synthesized proteins, enzymes, nucleic acids
DNA replication	Enzymes, nucleic acids
Membrane functions	Transport proteins, lipids

Source: Adapted from G.W. Gould and N.J. Russell, Sulphite, in *Food Preservatives*, N.J. Russell and G.W. Gould, eds., pp. 72–88, © 1991, Aspen Publishers, Inc.

tion can be many-fold greater than that in the surrounding solution. This occurs, and can be explained, when there is a difference in pH between the solution and the cell cytoplasm. With an intracellular pH of about 6.5 in *S. cerevisiae*, only about 0.0015% of free sulfite will exist as SO_2.[43] If the external pH is below 6.5, then SO_2 will diffuse into the cell and dissociate into HSO_3^- and H^+, thereby allowing further diffusion down its concentration gradient until the SO_2 concentration is the same on both sides of the cell membrane. Accumulation of sulfite by *Saccharomycodes ludwigii* and *Zygosaccharomyces bailii* was shown to occur similarly to that in *Saccharomyces cerevisiae*, resulting mainly from passive uptake of SO_2.[59,79] However, accumulation of sulfite in some other organisms appears to differ from this simple diffusion system. For example, studies of sulfate transport by *Candida utilis* indicated a common, active, energy-dependent transport system for sulfate, sulfite, and thiosulfite.[10,27] Sulfate analogues, including molybdate, selenate, and chromate, competitively inhibited the uptake of sulfate, as did sulfite and thiosulfate. Likewise, sulfite and thiosulfate inhibited sulfate uptake by *Penicillium chrysogenum*.[96] Selenate-resistant mutants of *C. utilis* had a defective transport system for sulfate, sulfite, and thiosulfate.[27] Although not being an important target for preservation by sulfite, *Desulfovibrio desulfuricans* was shown to possess a sulfite transport system[19] and mycelia of *Penicillium* and *Aspergillus* species were reported to transport sulfite.[89]

Therefore, with respect to transport of sulfite into microbial cells, it appears that free diffusion of molecular SO_2 is the major mechanism in those yeasts that are the most important targets for sulfite in food preservation, but alternative transport systems do occur in other organisms.

Interaction of sulfite with components of the plasma membrane have seldom been reported, though Stratford[78] did find that sulfite interfered with the accumulation of glucose and the amino acids arginine and lysine by *S. cerevisiae*. While this may have resulted from interaction of sulfite with a membrane protein, it is more likely that it resulted from collapse of the proton motive force.[73]

Having entered the cytoplasm, by whatever means, and been concentrated there (as explained above), the range of important molecules available for reaction with sulfite is high, so that many reactions that could lead to inhibition or cell death are possible, in addition to the acidification of the cytoplasm that will eventually occur if the external pH is sufficiently low. For example, it has been shown that just prior to cell death caused by sulfite, there is a rapid fall in ATP levels and a rise in free phosphate in *S. cerevisiae*.[74,75] Glyceraldehyde 3-phosphate dehydrogenase was shown to be the most sensitive enzyme on the Embden-Meyerhof-Parnas pathway, which is responsible for substrate level generation of ATP in *S. cerevisiae*.[38] Purified ATPase from yeast mitochondria was shown to be particularly sulfite sensitive.[47]

Different species of yeasts vary greatly in their resistance to sulfite. For instance, *Z. bailii*[85] and *Saccharomycodes ludwigii*[79] are much more resistant than *Saccharomyces cerevisiae*, which is generally regarded to be relatively sulfite tolerant, while *Kloekera apiculata* and *Hansenula anomola*[92] are much more sensitive. The sensitive strains are inhibited by about 0.2 to 1 mg l^{-1}, whereas some of the most resistant types tolerate up to about 20 mg l^{-1}. The reasons for these differences are not fully understood, but yeasts differ in their ability to bind sulfite[62] and binding by excreted acetaldehyde has been known for many years and has been proposed to play a role.[53,79]

While most of the antimicrobial applications of sulfite are directed at yeasts and molds in fruit and vegetable products and drinks at pH values well below neutrality, a small number of applications are directed at bacteria in raw meat products. These applications are primarily concerned with inhibiting the growth of spoilage bacteria, and consequently, extending shelf life. However, because these products, unlike many of the lowered a_w, low pH foods and drinks, have high water activities and pH values, an additional role of sulfite not found in most other applications is to improve safety by inhibiting the growth of any food poisoning bacteria that may be present. Sulfite is effective in this role because, although most active at low pH, it continues to influence the growth of microorganisms, including some types of bacteria, at pH values well above those typical of fruits and drinks and within the range typical of unfermented raw meat products.[11,20]

For example, the pioneering work of Dyett and Shelley[24] first showed that the predominant effect of adding sulfite to fresh sausages was to bring about a major change in the types of microorganisms that were able to grow. This, and later work, particularly of Banks and Board,[7] clearly demonstrated that sulfite caused a shift away from the Gram-negative–dominated flora of unpreserved meat and toward a Gram-positive flora largely consisting of *Brochothrix thermosphacta*, lactic acid bacteria, micrococci, and yeasts. The Gram-positive flora grew more slowly than the Gram-negative flora, so that the keepability of the fresh sausage increased when sulfite was added.

The general influence of sulfite and temperature together on spoilage flora are well illustrated by the data in Table 9-5. In Table 9-6, it is clear that even at a temperature as high as 22°C, a strong antimicrobial effect of sulfite is still noticeable.

The improvement of safety that derives from the use of sulfite in meat results first from the shift in flora from the Gram-negative types, that include contaminants such as *Salmonella* and *Escherichia coli*, and second from the effect of temperature. This was shown most convincingly by Banks and Board,[7] who conducted studies with *Salmonella*-inoculated sausages that were made with and without added sulfite and stored at different temperatures (Table 9-7). The first part of the table shows that Enterobacteriaceae were substantially inhibited in the sulfited products at 4°, 10°, and 15°C for at least 8 days, whereas growth occurred in the expected temperature-dependent manner in sulfite-free samples, even at 4°C. The second part of the table shows even more dramatic effects on the growth of *Salmonella virchow*, which did not multiply at 4° or 10°C, but at 15° and 20°C it was completely prevented from growing by sulfite, even though counts in the sulfite-free controls reached over 10^6 g^{-1} in 4 days at 15°C and in 2 days at 20°C. A similar pattern of responses was reported by Moerman et al.[51] using *S. typhimurium* and *E. coli.*

9.3 NITRITE

9.3.1 General

Preservation of meat and fish by reduction in water activity has been practiced since ancient times by drying or by the addition of solutes. Sodium chloride is the most-used solute for this purpose in the process known as curing. When naturally occurring deposits of salt containing nitrate as an impurity were used for curing, the nitrate became reduced to nitrite by microbial action, mainly due to micrococci and, in meat, reacted with the heme pigments to generate pink color. Later, naturally occurring deposits of nitrate (saltpeter; niter) were used deliberately. The origin of the use of nitrite itself is unclear, but followed the discovery prior to 1900 that it was the chemical species responsible for color development in cured meats. This resulted from its reaction with myoglobin and its derivatives to form nitrosomyoglobin.[56]

Today nitrite itself is therefore generally employed, usually mixed with sodium chloride in fixed proportion in "curing salts" so that variability in nitrite formation from nitrate is avoided. For instance, in Europe, while sodium nitrate (E251) and potassium nitrate (E252) can be used alone or mixed with sodium chloride, potassium nitrite (E249) and sodium nitrite (E250) can only be used mixed with sodium chloride.[61]

While curing has been widely accepted as a useful and safe procedure for many years, the demonstration in the late 1960s that potentially carcinogenic nitrosamines could be generated by reaction of nitrite with amino acids and amines in nitrite-containing foods, refocused attention on the role of nitrite, and on the possibility of finding alternatives for its various functions.[13] These concerns have led to reductions in the allowed nitrite input levels to foods from the levels of 200 mg kg^{-1} for many types of products toward the minimum required for satisfactory development of color, ie, probably about 20 mg kg^{-1} for most meat products.[95] For instance, in the US, maximum input levels are 156 mg kg^{-1}. Following the recommendation of a scientific panel, the US Department of Agriculture (USDA) restricted sodium nitrite to 120 mg kg^{-1} or potassium nitrite to

Table 9-5 Effect of Added Sulfite on Microbial Growth in Raw Pork and Beef Sausage

Initial Concentration of Sulfite (SO_2; mg kg^{-1})	*Days To Reach an Aerobic Count of 10^8 g^{-1} at (°C)*				
	−2	*0*	*3*	*10*	*16*
0	15	9	7	4	2
250	21	12	6	3	2
500	>27	>27	15	12	5
750	>27	>27	21	>12	7

Source: Reprinted with permission from J.G. Banks and R.G. Board, Sulphite Inhibition of Enterobacteriaceae Including Salmonella in British Fresh Sausage and Culture Systems, *Journal of Food Protection*, Vol. 45, pp. 1292–1297, © 1982, International Association of Milk, Food, and Environmental Sanitarians, Inc.

Table 9-6 Effect of Added Sulfite on Microbial Growth in Fresh Pork Sausage at 22°C

Incubation Time at 22°C (days)	*Total Aerobic Count (millions g^{-1})† in Sausages Containing Metabisulfite, Added at (SO_2; mg kg^{-1})*				
	0	*150*	*300*	*450*	*600*
1	136	78	2	3	0.2
2	256	243	73	5	2
3	608	691	445	62	39

†Initial count was 0.2×10^6 g^{-1}.

Source: Reprinted with permission from E.J. Dyett and D. Shelley, The Effects of Sulphite Preservative in British Fresh Sausages, *Journal of Applied Bacteriology*, Vol. 29, pp. 439–446, © 1966, Blackwell Science Ltd.

Table 9-7 Growth of Enterobacteriaceae and *Salmonella virchow* in Sulfited and Unsulfited Fresh Pork Sausage Meat

		Microbial Numbers (millions g^{-1})† after Incubation for (days)				
Temperature (°C)	*Sulfite (SO_2; mg kg^{-1})‡*	*1*	*2*	*4*	*6*	*8*
Enterobacteriaceae						
4	0	0.03	0.2	0.1	1	200
	600	0.03	0.01	0.008	0.01	0.01
10	0	0.06	2	3	1000	1000
	600	0.02	0.02	0.01	0.01	0.01
15	0	0.06	20	2000	2000	300
	600	0.02	0.02	0.008	0.03	0.02
20	0	0.05	200	2000	300	60
	600	0.04	0.04	0.006	0.6	3
Salmonella virchow						
4	0	0.004	0.006	0.006	0.006	0.004
	600	0.002	0.006	0.004	0.004	0.003
10	0	0.005	0.01	0.01	0.01	0.01
	600	0.002	0.005	0.004	0.004	0.005
15	0	0.005	0.04	1	2	2
	600	0.002	0.006	0.006	0.006	0.005
20	0	0.004	2	10	1	0.1
	600	0.002	0.006	0.006	0.002	0.003

†Initial numbers of Enterobacteriaceae were about 2 × 10 g^{-1}; initial numbers of *Salmonella virchow* were about 8 × 10 g^{-1}.

‡SO_2 added at 600 mg kg^{-1} reduces to about 450 mg SO_2 kg^{-1} free in the manufactured product.

Source: Reprinted with permission from J.G. Banks and R.G. Board, Sulphite Inhibition of Enterobacteriaceae Including *Salmonella* in British Fresh Sausage and Culture Systems, *Journal of Food Protection*, Vol. 45, pp. 1292–1297, © 1982, International Association of Milk, Food and Environmental Sanitarians, Inc.

140 mg kg^{-1} in bacon (with 550 mg kg^{-1} ascorbate or erythorbate), and nitrate was no longer permitted. In Europe, levels depend on product type[13] (Chapter 18). Legislation varies greatly in other countries. For example, the use of nitrate and nitrite in foods has been almost abolished in Norway. In 1975 Canada banned the addition of nitrate to all but a few products, resulting in a five-fold decrease in residual nitrite in cured meats between 1971 and 1978.[5] In the United Kingdom (UK), a maximum residual concentration of 175 mg kg^{-1} is specified for nitrite in bacon, and for other cured meat products and canned meat products an indicative ingoing amount of 150 mg kg^{-1} and a maximum residual concentration of 100 mg kg^{-1} are specified, all as sodium nitrite.[77] In a survey in 1996–97 of products from retail outlets in England and Wales, in general the concentration of nitrite in bacon ranged from <0.2 to 120 mg kg^{-1} and that in other meat types ranged from <0.2 to 170 mg kg^{-1}.[46]

9.3.2 Uses in Foods

While nitrite is mainly used in meat curing, the addition of nitrate during the manufacture of certain cheeses has been practiced for nearly 200 years.[59] As in the earlier forms of meat curing, this leads to the generation of nitrite *in situ*, which prevents "late gassing" caused by *Clostridium tyrobutyricum* and *C. butyricum* in the stored product.[44] The procedure is widely used in Europe and, more recently in Canada,[5] though not in the US.

The range of cured meat products has expanded over the years to include unfermented raw meats, fermented raw meats, dry cured meats, cooked (pasteurized) meats, and canned products (see Chapter 18). They have in common the characteristic flavor resulting from the raised levels of salt and also, to some extent, from the nitrite,[34] the characteristic color, and the suppression of microorganisms that require a high a_w for growth. This results, in general due to the inhibitory effects of salt, in the selection of nonproteolytic microorganisms so that it is typical of these products that shelf life is extended and putrefaction is rare.[71,95]

The major reactions of nitrite, particularly with heme compounds,[9] have been comprehensively reviewed.[15,56] Effects on cured meat flavor were reviewed by Gray et al.[34] Several reviews address microbiological aspects relevant to cured meat products,[39,60,71,76,86,95] and to fish.[26]

9.3.3 Chemistry of Nitrite

Nitrite is the anion of a weak acid (nitrous acid), which has a p*K*a of 3.36.

$$HNO_2 \rightleftharpoons H^+ + NO_2^-$$

Most nitrite-containing foods have pH values within the range from about 5 to about 6.5, so that the concentration of free acid to be expected at equilibrium will be as low as 0.1% to 1% or so of the added nitrite.

While the detailed chemistry of color development during meat curing is complex, the main nitrosating reactions are well known, and the principal reactant is dinitrogen trioxide, which is the anhydride of nitrous acid:

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

Reductants such as ascorbate or erythorbate, which are often added to cured products, accelerate color development by reacting with dinitrogen trioxide to produce nitric oxide:

$$N_2O_3 + HR \rightleftharpoons RNO + HNO_2$$
$$RNO \rightleftharpoons R + NO$$

where *R* is the reductant molecule.

Meat initally becomes brown when nitrite is added to it as heme compounds are oxidized. For example, purple-colored myoglobin (Mb) or red-colored oxymyoglobin (MbO_2), both containing iron in the ferrous state, are oxidized to brown-colored metmyoglobin, containing iron in the ferric state ($MbFe^{III}H_2O$). This may then react to form nitrosylmetmyoglobin ($MbFe^{III}NO$), which autoreduces to give the more stable Fe(II) form, nitric oxide myoglobin, or nitrosyl myoglobin (NOMb). This is the compound that gives the characteristic pink color to raw cured meats.[56] When cured meats are heated, the protein (globin) moiety in the myoglobin derivatives becomes denatured and detaches from the heme iron atom. The resultant color, typical of cooked cured meats, is then nitrosylmyochromogen (or nitrosyl protohaem). While these are the basic mechanisms of color formation, the details are complex, and new reactions are still being discovered.

Apart from the important color reactions, other reactions take place, some of which may be more significant microbiologically. Nitrite has an antimicrobial effect (Section 9.3.4) that increases as the pH is reduced so that, as with many weak acids, the unionized nitrous acid has been implicated as the active species since first proposed 70 years ago by Grindley.[33] However, antimicrobial effects additional to those attributable to HNO_2 have been often reported. In particular, heating of nitrite in bacteriological media produces a wide variety of products, including some that are much more antimicrobial than nitrite itself, first recognized by Perigo et al.,[58] and referred to as the "Perigo factor." Some of the compounds, particularly those derived from reactions between nitrite, cysteine, and ferrous ions, including nitrosyl-cystyl ferrate and more complex "Roussin black salts," were shown to be strong inhibitors of growth from spores of *Clostridium* species. Many other antimicrobial products, such as cysteine nitrosothiol,[40] have been described.[52] Some of these, while detectable and effective in culture media, were found to be much less easily detectable in foods[91] so that their importance in the microbiological stability of cured products remains doubtful. On the other hand, if some of these substances are deliberately added to foods (eg, S-nitrosocysteine to cured, pasteurized ham[90,91]), inhibition of growth from spores of *Clostridium* species could be demonstrated.

The problem of recognizing specific nitrite reaction products and assigning them particular antimicrobial roles has therefore been two-fold. First, the reaction products are numerous. Cassens et al.[14] used ^{15}N-nitrite in an attempt to identify the components of meat that reacted with nitrite. They failed to account for about 20% to 30% of the nitrite that was added. The remaining 70% to 80% had reacted with myoglobin (5% to 15%), sulphydryl-containing molecules (5% to 15%), lipids (1% to 5%), and proteins (20% to 30%). Some was lost as gas (1% to 5%), while 1% to 10% became oxidized to nitrate, and only 5% to 20% remained as nitrite. Second, antimicrobial activities of products reported to be generated in bacteriological media containing nitrite do not appear to be produced to the same extent in foods.[4]

9.3.4 Antimicrobial Activity of Nitrite

The antimicrobial activity of nitrite and its increased effectiveness with reduction in pH, first reported in the 1920s,[33] was confirmed and extended in more detailed studies by Tarr[82–84] and others, eg, for *Staphylococcus aureus*,[16] *Bacillus* spores,[25] and *Clostridium* spores.[58] At inhibitory concentrations, nitrite prevents outgrowth from spores rather than arresting their germination (eg, for *Bacillus* spores including *B. cereus*, *B. subtilis*, *B. polymyxa*, *B. coagulans*, and *B. licheniformis*) at concentrations of about 200 mg L^{-1} at pH 6[29] and for *Clostridium* species (eg, *C. sporogenes*[21,22]). It is interesting that nitrite can also actually cause spores to germinate, probably by cleaving bonds in peptidoglycan in the spore cortex, but only at higher concentrations and/or lower pH values than occur in foods, eg, thousands of mg L^{-1} at pH values less than 6,[23] so its practical relevance is doubtful. While effective minimum inhibitory concentrations for most organisms were about 100 to 200 mg kg^{-1} at pH 6 (eg, for species of *Achromobacter, Aerobacter, Escherichia, Flavobacterium, Micrococcus,* and *Pseudomonas*[84]), *Lactobacillus* and *Salmonella* were more tolerant,[16] as was *C. perfringens* when compared with other clostridia.[57]

The way in which nitrite interferes with the growth of microorganisms was reviewed by Woods et al.[95] They showed that it causes a rapid decrease in intracellular ATP levels. There is persuasive evidence that it brings about this decrease mainly by inhibition of the phosphoroclastic system in *C. sporogenes* and in *C. botulinum* A and B (proteolytic and nonproteolytic strains), C, D, and E.[94,95] This probably occurs somewhat analogously to the meat curing reactions, by reaction of nitrite with nonheme iron proteins such as the ferredoxins that play a key role in this system (eg, in *C. botulinum*[63]). Action of nitrite on the phosphoroclastic system was enhanced by ascorbate, and nitrite plus ascorbate was shown additionally to inhibit a wide range of 2-oxo acid oxidoreductases.[95]

Bearing in mind that (1) input levels of nitrite in most countries do not exceed about 200 mg kg^{-1}, (2) losses occur by reaction with food components, and (3) in many cured meat products the pH is above 6, the importance of nitrite as an antimicrobial in foods has often been questioned. In cheeses, in which the pH is low, the efficacy of nitrite as a spore-control agent is, of course, not doubted. In fermented meats, likewise, antimicrobial activity should be expected. This may be important in the inhibition of environmental contaminants such as *Listeria monocytogenes.*[12,41,42,50] This is well illustrated by the data in Table 9-8, in which the effects of combinations of salt, nitrite, and pH on growth of *L. monocytogenes* are compared. Even at concentrations up to 100 mg kg^{-1}, little inhibition is evident at pH 6. However, at pH 5.3, substantial inhibition is shown at 50 mg kg^{-1}. This combination of salt, pH, and residual nitrite is relevant to a number of salami and similar fermented meat products.[42,43] Likewise, studies with *S. aureus* found no inhibition of growth and enterotoxin A production by 200 mg kg^{-1} at pH 6.6.[48] *S. aureus* is capable of growth and enterotoxin formation aerobically in nitrite-containing sausages and bacon,[18,28] but inhibition becomes apparent as the pH is reduced.[87] Whereas the antimicrobial effects of nitrite are therefore well established, and may be important in the preservation and safety of the lower pH foods, their relevance in higher pH foods has been less clear. In particular, attention has centered on the possible role of nitrite in the prevention of outgrowth from spores of *C. botulinum* in vacuum packed cured products, raw meat products (such as bacon), and heated products (such as cooked hams and nitrite-containing sausages).

Table 9-8 Influence of Nitrite Concentration on Growth of *Listeria monocytogenes* in Tryptone Soy Broth at 25°C

		Time to Growth (hours)† *at Nitrite Concentration (mg kg^{-1}) of*			
pH Value	*NaCl (% w/v)*	*0*	*50*	*100*	*200*
6.0	0.5	19	19	19	22
	2	19	19	19	22
	4	19	20	22	25
	6	20	23	24	43
	8	43	43	43	68
5.3	0.5	19	28	49	190
	2	19	28	118	NG‡
	4	21	45	118	NG
	6	28	NG	NG	NG
	8	45	NG	NG	NG

†Inoculum, 4 × 10^3 ml^{-1}; growth, 10^6–10^7 ml^{-1}.

‡NG = no growth in 21 days.

Source: Reprinted with permission from P.J. McClure, T.M. Kelly, and T.A. Roberts, The Effects of Temperature, pH, Sodium Chloride and Sodium Nitrite on the Growth of *Listeria Monocytogenes, International Journal of Food Microbiology,* Vol. 14, pp. 77–92, © 1991, Elsevier Applied Science.

A series of studies by Roberts et al.[66–69] investigated the combined effects of pH, salt, nitrite, and ascorbate in pork slurry on the probability of growth from spores of *C. botulinum*. The study led to a mathematical model for predicting the effect of these parameters on *C. botulinum* growth.[72] The most important factors influencing growth from spores were pH value and salt concentration. Other food systems studied have included vacuum packed cured meats and liver sausage, pasteurized cured comminuted pork, and shelf-stable canned cured meats. Hauschild et al.[36] summarized data from these numerous product-specific *C. botulinum* challenge tests and tried to estimate the degree of safety contributed by nitrite on a "probability of growth from one spore" basis. The influence of nitrite could be seen clearly in many of the experiments considered. A particularly striking example was given by Tompkin et al.,[88] who inoculated cured pork with spores of *C. botulinum* A and B while arranging that the same *input* levels of nitrite would deliver different *residual* levels. This was done by adding extra hemoglobin to react with, and therefore reduce, residual nitrite levels when required. The results (Table 9-9) showed that the highest residual levels delivered the most effective inhibition, whereas the lower residuals were less inhibitory, in order of decreasing concentration and whatever the input level. Pasteurization in these experiments was at 74°C. With more severe heating, there is ample evidence that spores that survive the heat process are injured. Injury is expressed as increased sensitivity to a variety of antimicrobial agents and to environmental parameters that are not optimal for growth.[1,30] Nitrite is among the antimicrobial agents that become more inhibitory following heat damage to spores. For example, heat treatment of spores of *C. sporogenes* PA 3679 at 115°C for 5 minutes caused about a 10^2-fold decrease in colony count as estimated by plating on a nutrient agar at pH 6.8, while addition of 50 mg L^{-1} of sodium nitrite to the medium resulted in a 10^5-fold decrease.[70] After heating at 90°C for 6 hours, growth from spores of *C. perfringens* was much more sensitive to inhibition by nitrite as well as by salt than was growth from spores that had simply been heat activated.[17] Lucke[45] reported that *D*-values of *C. botulinum* types A and proteolytic B at 100° and 105°C were much longer in liver sausage mixture (prepared from precooked ingredients) than in Bruhwurst (made from raw ingredients only). Nitrite did not prevent development of the surviving spores in the liver sausage, but was markedly inhibitory in the Bruhwurst. It was concluded that fat in the liver sausage protected spores from heat injury and, as in the studies of Tompkin et al.,[87] that nitrite was inactivated by iron derived from the liver.

Many such cured meat products, especially liver sausage and blood sausage, would become unacceptable to consumers if they received a full botulinum cook of F_0 2.5. Shelf-stable cured meat products such as canned luncheon meats, hams, and sausages in hermetically sealed containers are therefore usually heated only to F_0 0.2 to 1.0 or so. They rely for their stability and safety on combinations of the low incidence of spores in the raw materials (numbers of *C. botulinum* spores in 138 samples of Canadian liver sausage[35] were estimated to be about 0.15 kg^{-1}), heat damage to any spores that are present, and their sensitivity to inhibition by salt and nitrite.[45,86]

While such experiments show that nitrite does have an antibotulinal effect, and under the conditions relevant to cured meats, its quantification and extrapolation to practical issues of food safety have been less easy to make. For example, while the experiments summarized in Table 9-9 show that without doubt, by moving from 112 to 84 to 30 to 6 mg kg^{-1} of nitrite in the particular pasteurized ham, the probability of spore outgrowth is increased, even the highest levels of nitrite did not completely prevent outgrowth of all the 10,000 or so spores that were originally present in each can and so did not deliver the equivalent of a classical 12-D process.

Hauschild et al.[36] summarized the implications of such observations by concluding that (1) the degree of safety of the cured products that had been studied differed by several orders of magnitude and (2) large reductions in the concentrations of nitrite in the products could have severe consequences. Hauschild and Simonsen[37] further concluded, "Research data are limited and do not allow us at present to propose minimal requirements of the essential safety factors. Instead, our risk assessment relies heavily on commercial practice and experience."

At the request of the USDA, the US National Academy of Sciences considered the health and

Table 9-9 Influence of Residual Nitrite Levels on Inhibition of Growth from Spores of *Clostridium botulinum* in Pasteurized Cured Pork

Initial $NaNO_2$ (mg kg^{-1})	*Hemoglobin Added (%)*	*Residual Nitrite (mg kg^{-1})*	*Swollen Cans (25 per variable) after (days)*[†]				
			20	*40*	*60*	*80*	*100*
156	0	112	1	2	2	2	13
156	1	84	1	1	3	16	25
50	0	30	3	4	19	25	25
50	1	6	25	25	25	25	25

[†]The inoculum consisted of 10^2 spores of *C. botulinum* A (5 strains) and proteolytic B (5 strains) per g in pasteurized cured pork in cans. Salt concentration was 3.53% to 3.98%. Pasteurization was in 77°C waterbaths to a center temperature of 74°C. Incubation was at 27°C.

Source: Reprinted with permission from R.B. Tompkin, L.N. Christiansen, and A.B. Shaparis, Cause of Variation in Botulinal Inhibition in Perishable Canned Cured Meat, *Applied Environmental Microbiology*, Vol. 35, pp. 886–889, © 1978, American Society for Microbiology.

safety aspects of dietary nitrate and nitrite and possible nitrite alternatives.[5,6] It was concluded that "it is not possible to estimate the potential morbidity or mortality from *C. botulinum* if nitrite were to be removed as a curing agent from certain products" and "in view of the possible but unquantified risk resulting from the use of nitrite as a curing agent, the search for alternatives to the use of nitrite should continue." It is because of all these considerations that there remains a well-founded reluctance to accept further relaxation of the basic preservation systems, including the contribution made by nitrite, that operate in cured meats.

Nitrite alternatives that have been proposed have included the addition of other antimicrobials such as sorbate, alone or with reduced levels of nitrite.[49,64] Other approaches, particularly in Canada, have aimed to deliver the necessary color to cured products by the addition of preformed heme-nitrite reaction products[54] although, while reducing nitrite levels, this sort of approach leaves open the microbiological consequences.

Following a USDA initiative to reduce input levels of nitrite in bacon to a maximum of 120 mg kg^{-1}, and to require the addition of 550 mg kg^{-1} of ascorbate or isoascorbate, a novel approach was developed. In this approach (the "Wisconsin process"), a combination of 40 to 80 mg kg^{-1} of sodium nitrite and a readily fermentable carbohydrate (minimum 0.7% sucrose) plus an acid-producing culture (eg, *Pediococcus acidilactici*) is added,[81] so that pH is reduced, enhancing the effectiveness of the nitrite that is present and, should the product be stored too long or at too high a temperature, further acidification is ensured.

9.4 CONCLUDING REMARKS

Sulfite and nitrite both deliver benefits to foods that do not rely on their antimicrobial activities, such as the enhancement of flavor, color, and antioxidant activity. However, they are the only inorganic preservatives that have clear antimicrobial activities as well. Both are most effective at low pH values, and this is widely exploited in the case of sulfite in a variety of acidified products, but is less relevant for nitrite, where most of the applications are in foods that are not substantially acidified. Partly for this reason, sulfite plays a much more obvious role than nitrite as an antimicrobial preservative.

Both anions have been associated with undesirable potential toxicological effects, so that there has been interest in reducing their levels or eliminating them from foods and in finding alternatives for them. However, to some extent because of the multifunctionality, widely effective alternatives have been difficult to find. At the same time, reductions in levels have been approached with caution because of the possibility of increased risk of food poisoning. This is so for the use of sulfite in meat products in some countries, but more so with respect to the antibotulinal role of nitrite in cured meat products that, although there are still conflicting data, most authorities agree it would be unwise to lose.

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10 Modified Atmospheres

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10.1 INTRODUCTION

10.1.1 General

The shelf life of food can be enhanced by modifying the gas composition of air, ie, increasing or decreasing the oxygen content and/or increasing the level of carbon dioxide. Nitrogen levels may be varied to displace oxygen or to act as an inert filler that keeps flexible packages from collapsing but has no biological activity.

It is understandable that limiting access to oxygen will affect aerobic organisms, however, as early as 1877, microbiologists had demonstrated the inhibitory effects of carbon dioxide on microorganisms.[127] During the period from 1880 to late 1920, about one hundred reports were published within this field.[153] The following was concluded: (1) high pressures of carbon dioxide can inactivate some types of microorganisms; (2) high concentrations of carbon dioxide, at normal atmospheric pressure, can be inhibitory for many types of microorganisms, though the sensitivity to carbon dioxide varies between wide limits; and (3) increased concentrations of carbon dioxide are required for growth of certain organisms.

The findings that the growth-inhibitory effects were different for different organisms, confirmed by Coyne[22] and Haines,[73] is important for an understanding of food spoilage flora under modified atmospheres. Hence, modified atmospheres not only reduce microbial growth rates, but also they may impose changes in the composition of the microbial flora and may even improve the growth of some microaerophiles. This chapter will deal mainly with the controlling effect of modified atmospheres on the composition of the microflora, ie, on the types of microorganisms that make up the dominant spoilage microflora under different storage conditions.

10.1.2 Terminology and Technique

Storage of food under modified atmospheres (MA), ie, an atmosphere where the ratios between the air components have been changed (modified atmosphere packaging [MAP]), can be achieved in several ways. Usually the term modified atmosphere implies the use of some form of package based on a laminated plastic film with a substantial head space, at least at the moment of packaging. However, a "vacuum package" also provides a modified atmosphere; air is removed and the head space is decreased. In this limited space the product and/or the metabolic activities of contaminating microorganisms change the proportions of oxygen and carbon dioxide. To distinguish between a vacuum package where the atmospheric modification is passively created, and the type of package where gas has been deliberately flushed into the package, the term "gas package" has been used. However, the term is mostly avoided in the industry because of the possible negative implications.

The permeability of the packaging film to different gases is of major importance for the efficiency of the procedure. Many films of different qualities are on the market but they are beyond the scope of this discussion.[117,123,125] The most gas-impermeable materials available are those that include a layer of aluminum in the laminate.

The atmosphere in vacuum packages or modified atmosphere packages can be controlled by the use of oxygen absorbers or carbon dioxide emitters (active packaging). These techniques can also be used to introduce or withdraw other components in the package, eg, ethylene, which affects the ripening of fruits and vegetables, or ethanol, which suppresses mold growth and retards the staling of bread. The industrial use of oxygen scavengers and ethylene absorbers is increasing, especially in Japan and the United States (US).

Another technique for changing the atmosphere around food is to change and maintain the atmosphere directly in a storage room or in a larger container. This is done by continuously controlling the gas composition, the so-called controlled atmosphere (CA) storage. This procedure was commercially applied for fruits as early as 1928.[143]

Another early application of the concept of changing the gas environment was the carbonation of drinking water.[51,80] It was found that carbonation increased both the shelf life and the safety of the product.[153] In the 1950s, interest in using high carbon dioxide pressures for the inactivation of microorganisms was pursued.[77,78] This concept has been taken up again, and is now supported with new techniques that make it feasible to treat food at very high pressures (5–35 MPa).[2,155] Five atm (505 kPa) of carbon dioxide extended the time to reach 10^6 colony-forming units per cm^2 of pork by about three times compared with storage under 1 atm (101 kPa) of carbon dioxide, at 4°C.[8] The increased pressure had no significant effects on the composition of the spoilage flora.[8] However, this chapter will be limited to modified atmospheres at atmospheric pressure provided by current packaging techniques and employing flexible packages.

10.2 THE EFFECT OF OXYGEN ON MICROORGANISMS

Oxygen is toxic to all life provided the partial pressure is high enough. Explanations of its toxicity include the following: (1) inactivation of certain enzymes, (2) increasing intracellular generation of hydrogen peroxide, (3) oxidation of membrane lipids, and (4) *in vivo* production of the superoxide radical. These effects may coincide but formation of superoxide radicals is probably of major importance.[74] For example, oxygen resistance of cells can be correlated with their superoxide dismutase activity.[70,104] Dismutation of superoxide radicals gives rise to hydrogen peroxide that, if not rapidly destroyed by catalase, in turn, can react with further superoxide radicals, resulting in the formation of extremely reactive compounds, such as the hydroxyl radical.

Different types of organisms are protected to various degrees against the oxygen radicals. Superoxide dismutase is important for protection but simultaneous action of other defense enzymes, including catalases and peroxidases, is required for removal of the hydrogen peroxide formed in the dismutation reaction.[76] Most aerobic and facultatively aerobic microorganisms can endure the partial pressure of oxygen in air, but microaerophilic organisms can be inhibited by this concentration of oxygen. The partial pressure of oxygen in air is usually toxic for anaerobic organisms that lack efficient systems to get rid of the oxygen-generated radicals.

Organisms able to utilize oxygen have an ecological advantage. Oxygen metabolism results in a high energy yield. Thus, the toxic effects of oxygen are, in practice (ie, up to 100% of the gas phase under atmospheric pressures), of minor importance in connection with microbiological food spoilage. More important is the growth-supporting effect of oxygen because organisms with aerobic metabolism, in the presence of oxygen, always will outgrow those lacking this ability under otherwise unrestricted conditions.

Pseudomonas, *Psychrobacter*, and *Shewanella* are aerobic genera, but they can also grow under anaerobic conditions provided that there is a suitable electron acceptor present. *Shewanella putrefaciens*, for example, is able to reduce trimethyl-amine-oxide in fish to trimethyl-amine.[93,96] Many bacterial taxa can grow in both aerobic and anaerobic environments, eg, *Enterobacteriaceae*, *Aeromonas*, *Photobacterium*, *Brochothrix*, and lactobacilli. Lactobacilli, including the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella*, differ within wide limits in their response to oxygen. The taxa that are microaerophilic compete best with other types of bacteria in the presence of traces of oxygen. Strictly anaerobic organisms (for example, *Clostridium* and *Bifidobacterium*) seldom dominate during food spoilage. Multiplication of clostridia to high numbers in food usually requires either that the normal spoilage flora has been killed (together with storage at anaerobic conditions), or that a heavy population of other organisms provide the anaerobes with an oxygen-free growth environment.

10.3 THE EFFECT OF CARBON DIOXIDE ON MICROORGANISMS

10.3.1 Mechanism of Effects

Carbon dioxide has an important regulatory influence in the living cell. All cells require carbon dioxide for growth, but if the partial pressure of carbon dioxide increases over a certain critical level, metabolic activity will be retarded.[15,22,127,153]

Several hypotheses to explain the growth-inhibitory effects of carbon dioxide have been put forward. Some examples are that carbon dioxide should: (1) cause a decrease of the intracellular pH,[22,158] (2) inhibit (or stimulate) enzymatically catalyzed reactions and enzyme synthesis,[32,87] and (3) interact with the cell membrane.[39,42,133] There are probably two major effects involved: (1) effects of both aqueous carbon dioxide and HCO_3^- on the state of the cell membranes and/or the function of the membrane proteins, and (2) effects on cytoplasmic enzymes. The former effects are probably the most important ones in those organisms that are relatively resistant to carbon dioxide (see below), ie, where a relatively high level of carbon dioxide is required for growth inhibition. However, for more sensitive organisms that are affected by small increases in the partial pressure of carbon dioxide, interference with enzymes and their activities is probably more important.[87] It has also been concluded that "most probably, a major factor in the efficacy of carbon dioxide lies in its ability to penetrate the bacterial membrane, causing intracellular pH changes."[32] Generally speaking, carbon dioxide inhibition of microbial growth cannot be attributed to a single target in the cell. Inhibition by carbon dioxide seems to be the overall result of a complex interaction with several key physiological reactions. The likely mechanisms involved in carbon dioxide inhibition have been thoroughly reviewed.[32,87]

10.3.2 The Effect of Concentration

The solubility of carbon dioxide in water is high. Carbon dioxide distributes approximately equally per unit volume between the gas phase and the water phase.[35] At moderate temperatures and below 1 atm (101 kPa), if the concentration of carbon dioxide in the gas phase is in equilibrium with the solution, the solubility follows Henry's law ($[CO_2]aq = k_H pCO_2$, where pCO_2 is the partial pressure in atm of carbon dioxide in the gas phase and k_H is the Henry's law constant in units of mol atm^{-1}). The proportionality of solubilities and partial pressures may be assumed under atmospheric pressure.[132] The concentration of carbon dioxide is often given as the percent of carbon dioxide in the gas phase at atmospheric pressure.

The carbon dioxide molecule can, in water, be hydrated to carbonic acid, and then further dissociated to bicarbonate and carbonate as follows:

$$CO_2 + H_2O + \mathbf{CO_2[H_2O]} \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

The ratio of these forms in the aqueous phase is dependent on several factors, including the pH. The hydration of carbon dioxide to H_2CO_3 takes time because of the rearranging of the valence bonds between the carbon and the oxygen, but the dissociation is quick.[35,72] The equilibrium of hydration ($\mathbf{CO_2[H_2O]} \leftrightarrow H_2CO_3$) is much displaced toward the unhydrated side, meaning that the hydrated form normally can be neglected, and the system of the carbonic acid is represented by **solvated** carbon dioxide ($CO_2[H_2O]$). The distribution of carbonate species as a function of pH is shown in Figure 10-1.

The retarding effect of carbon dioxide on bacterial growth increases along with increasing concentration[41,67] (Figure 10-2). In the figure the growth rate decreases throughout the range of carbon dioxide concentrations tested, but the decrease is steeper at the lower range of concentrations. This trend is also in agreement with other observations.[20,67,68]

Carbon dioxide resistance is different for different types of microorganisms. Examples of carbon dioxide–sensitive organisms are frequently found in the genus *Pseudomonas* and among many of the molds that are associated with food. Their growth is often significantly retarded at a partial pressure as low as 10 kPa (0.1 atm or 10% [v/v] carbon dioxide in the gas phase at atmospheric pressure).[54,99,109,156]

Bakers yeast (*Saccharomyces cerevisiae*), on the other hand, is unaffected by up to about 51 kPa (0.5 atm),[18] while the genus *Lactobacillus* is affected only slightly at 100 kPa of carbon dioxide (1 atm).[110] As much as 869 kPa (8.6 atm) of carbon dioxide was required to reduce the maximum specific growth rate of a strain of *Lactococcus lactis* subspecies *cremoris* by 50%.[41]

The growth resistances to carbon dioxide of some strains of different species are compared in Figure 10-3. The strains were tested under anaerobic conditions. Aerobically growing organisms seem often to be more sensitive to carbon dioxide than anaerobically growing ones.[41,68] However, anaerobic species do not necessarily possess higher resistance to carbon dioxide than facultatively anaerobic ones (Figure 10-3), and the resistance can differ between different strains of the same species.[86] Figure 10-3 demonstrates that there is no general difference in resistance between Gram-positive and Gram-negative genera as sometimes has been stated in the literature.[141,147] On the other hand, lactobacilli are relatively resistant to carbon dioxide, which is in accordance with observations from packaging experiments using modified atmospheres[23,82] (see below).

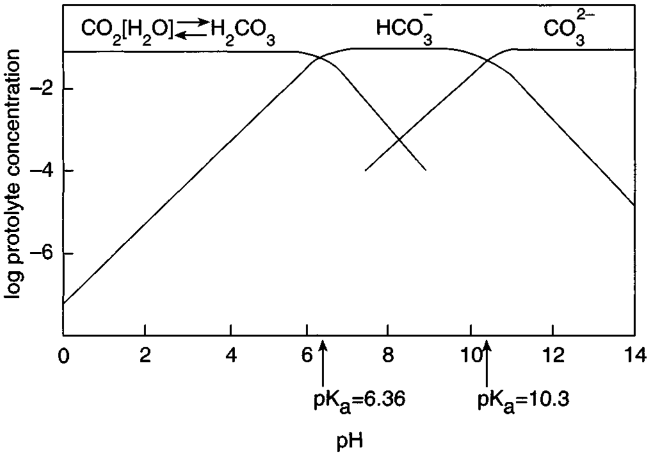

Figure 10-1 Fractions of H_2CO_3, HCO_3^-, and CO_3^{2-} As a Function of pH at 0.1 mol Carbonic Acid per L of Water. *Source:* Copyright © 1989, Göran Molin.

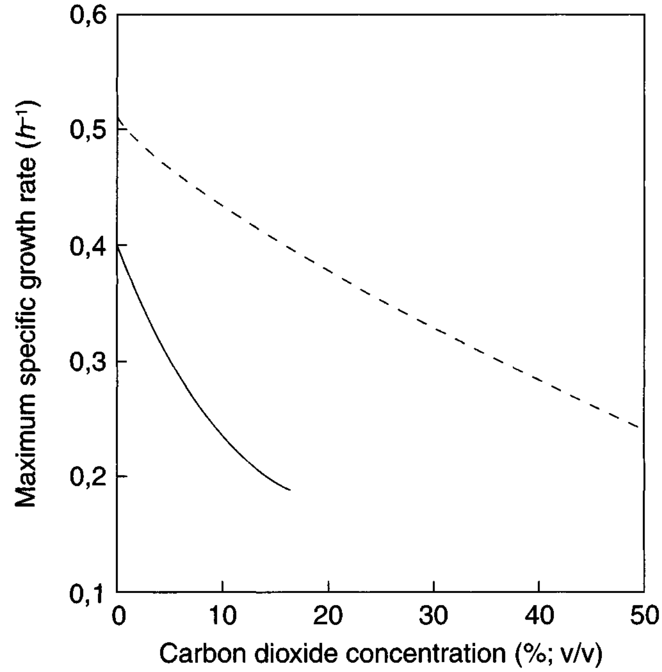

Figure 10-2 The Effect of Different Partial Pressures of Carbon Dioxide on the Maximum Specific Growth Rate of *Pseudomonas fragi* ATCC 7943 (Lower, Solid Line), Growing in a Fermenter at Constant pH (pH = 6.7; Temperature = 25°C) in a Complex Growth Medium, and the Meat Spoiling *Pseudomonas fragi* 72 (Upper, Hatched Line), Growing in a Fermenter at Constant pH (pH = 6.0; Temperature = 25°C) in a Synthetic Growth Medium with Citric Acid As the Only Carbon and Energy Source. The concentration of carbon dioxide is given as percentage of the gas phase; the fermenters were sparged with the gas mixture (sufficient oxygen for unlimited growth were present in all cultures). *Source:* Data from S-O Enfors and G. Molin, Effect of High Concentrations of Carbon Dioxide on Growth Rate of *Pseudomonas Fragi, Bacillus Cereus* and *Streptococcus Cremoris, Journal of Applied Bacteriology*, Vol. 48, pp. 409–416, © 1980, and G. Molin, Combined Carbon Dioxide Inhibition and Oxygen Limitation of the Growth of *Pseudomonas Fragi* 72 in Batch and Continuous Culture, *Journal of General Microbiology*, Vol. 129, pp. 2885–2891, © 1983.

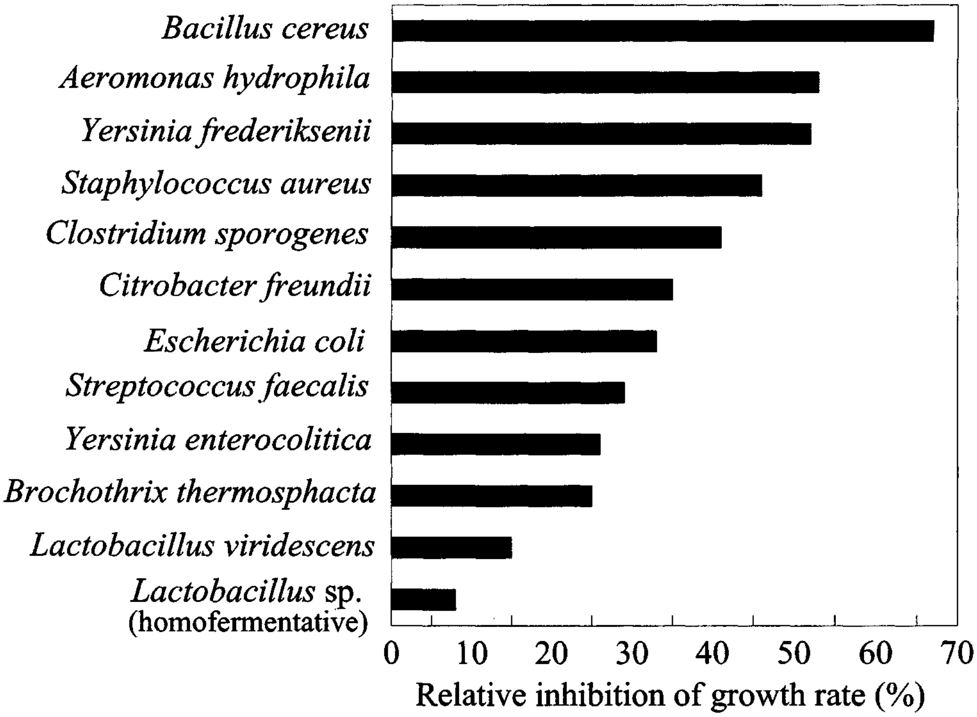

Figure 10-3 Relative Inhibition of Some Strains, Representing Different Species, by 101 kPa (1 atm) Carbon Dioxide, ie, 100% CO_2 (in Broth at pH 6.2; Temperature, 25°C). The relative inhibition was calculated as the ratio between the maximum specific growth rate in 101 kPa carbon dioxide and the maximum specific growth rate in 6 kPa (0.05 atm) carbon dioxide in nitrogen. *Source:* Copyright © 1989, Göran Molin.

10.3.3 The Influence of Temperature

The growth-inhibitory effect of carbon dioxide is strongly affected by temperature. The inhibitory effect of a certain partial pressure of carbon dioxide in the gas phase increases with decreasing temperature.[43,67] In the case of *Pseudomonas fragi* (strain ATCC 7943), this is due to the increase in water solubility of carbon dioxide at decreasing temperatures.[43] Thus, the solubility of carbon dioxide in water increases with decreasing temperature, and the vegetative microorganisms, mainly consisting of water, and enclosed by the water phase, are exposed to a higher carbon dioxide concentration when the temperature is low. This is also true if the bacteria are growing on a food surface, eg, a meat surface. Furthermore, there can be an additional inhibitory effect as a result of the lowered temperature itself. This was indicated for a mesophilic strain of *Bacillus cereus*, which had a carbon dioxide resistance about twice that of *P. fragi* ATCC 7943.[43] The additional effect must, of course, be dependent on how effectively the organism combats the effects of low growth temperatures. *P. fragi* grows relatively well down to the freezing point of the substrate. Thus, the shelf life of food under a carbon dioxide-enriched atmosphere is prolonged by low storage temperatures (see Figure 10-5, and below).

10.3.4 The Influence of pH

In water, the aqueous CO_2 molecules are in equilibrium with H_2CO_3, HCO_3^-, and CO_3^{2-}. The concentration of carbonate ions (CO_3^{2-}) at pH below 8.0 is too low to be of any biological significance and at pH below 6.0, the major portion of dissolved CO_2 exists as hydrated CO_2 molecules (H_2CO_3 constitutes only a small fraction of aqueous CO_2). Thus, there are two molecules to take into account, CO_2 and HCO_3^-. The former dominates at pH values below the pK_a (6.37) for aqueous CO_2/HCO_3^- and the latter above. Both CO_2 and HCO_3^- have biological effects but their relative inhibitory effects are not known.[32,87] However, there is evidence that CO_2 may have the greater effect on spore germination.[39]

The growth-retarding effect of carbon dioxide on *Pseudomonas fragi* (ATCC 7943), growing in fermenter cultures under continuous sparging with the tested gas mixtures, was not affected by pH in the range of 5.7 to 6.7.[43] On the other hand, the extension of shelf life due to vacuum packaging is lower for dark-cutting, high-pH beef (pH 6.2–6.7) than for normal beef (pH below 6.0).[46,128,150] This indicates that pH may affect inhibition by carbon dioxide in closed systems (packages) where the available amount of carbon dioxide is limited. A substantial amount of carbon dioxide is accumulated in a vacuum package of meat, and this carbon dioxide is the major reason for the shelf life prolonging effect (see below).

10.3.5 The Influence of Growth Medium

The growth-inhibitory effect of carbon dioxide in the gas phase is dependent on its solubility in the medium surrounding the microorganisms. The amount of carbon dioxide that can be dissolved in a medium decreases with decreasing water activity, and increases with increasing buffer capacity.[102] However, even though a lowered water activity reduces the amount of dissolved carbon dioxide in the medium, its antimicrobial action will enhance the growth-inhibitory effect of carbon dioxide.[6]

The inhibitory effect of carbon dioxide on the growth of organisms is influenced by the nutritional situation, for example, by the carbon source that is being utilized.[67,89] The catabolism of amino acids by *Pseudomonas* species was especially sensitive to increased levels of carbon dioxide while that of glucose was less affected.[111,148]

10.3.6 Additional Factors

A crucial factor in the use of modified atmosphere to prolong shelf life is that the product has a low contamination level at the time of packaging.[1,105]

A factor that will influence the change in gas composition in the package during storage, and hence the partial pressure of carbon dioxide in the package, is the head space–to–product volume.[159] The relevance of this factor must be considered for each individual type of application.

10.4 THE USE OF MODIFIED ATMOSPHERES IN PRESERVATION OF FRESH MEAT

10.4.1 Spoilage

The initial microflora of meat, for example, beef, lamb, pork, and poultry, always represents a complex mixture originating from skin and mucous membranes, and from environmental sources such as soil and water. Typical groups present are *Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Psychrobacter*, *Moraxella*, coryneforms, *Enterobacteriaceae*, *Staphylococcus*, and *Micrococcus*.[8,9,23,26,45,46] But even if the microbial variety is huge, the total numbers of organisms are usually relatively low. The environment then enforces a selection pressure on the microflora, and the types of organisms most fitted for the specific environment will outgrow the others, become dominant, and reach high numbers and spoil the meat. The microbial spoilage point of a food can be defined as "the time at which the activities of microbes present in a product become offensively evident to the senses."[62]

Irrespective of whether the meat originates from beef, lamb, pork, or poultry, the typical spoilage flora of refrigerated meat stored at 8°C or lower in air is composed mainly of *Pseudomonas*,[4,9,23,46,65,107,108] with *Pseudomonas fragi* as the most frequently dominating species, followed by *Pseudomonas lundensis* and certain biotypes of *Pseudomonas fluorescens* (especially biovariant 1).[23,115,116] Occasionally *Psychrobacter* (*Psychrobacter immobilis*) may reach significant numbers.[23]

The interior of fresh meat from healthy animals is almost free of bacteria and other microorganisms. The surface is where that the microorganisms multiply and reach numbers high enough to cause spoilage. As a general rule, meat acquires an offensive odor when the bacterial flora reaches about 10^7 colony forming unit (CFU) cm^{-2} of meat surface, and when the numbers have reached about 10^8 CFU cm^{-2} the meat surface becomes slimy.[23,64,83,112] At this point, the easily available nutrients on the surface become depleted, the microbial flora starts to become proteolytic and penetrates into the meat.[24,83] Before the bacteria attack the protein, they utilize more easily available carbon and energy sources; proteins must be hydrolyzed to amino acids before they can easily be utilized. Easily utilized compounds in meat, other than free amino acids (about 0.4% [w/w]) are, for example, lactic acid (0.9%) and glucose (0.01%).[60,111] In spite of these relatively low concentrations, the spoilage flora can be well supported up to around 10^7 CFU cm^{-2} due to diffusion of nutrients from the interior of the meat.[23]

Pseudomonas species that cause spoilage of meat have a comparatively high growth rate at refrigeration temperatures. On the meat surface, they primarily grow by using free amino acids, lactic acid, and glucose and, when they reach higher cell concentrations, they eventually produce lipases and proteases. Off-odors result primarily from the production of ethyl and methyl esters, short chain fatty acids, and sulfur-containing compounds.[23,36] From the bacteriological point of view, no general differences exist among the spoilage of meat from cows, pigs, lambs, and poultry. However, the shelf life can differ because of differences in contamination levels, the pH of the meat (the pH is relatively high for poultry: 5.7–6.7),[107] and the ratio between meat surface and volume, which is often high for poultry.

10.4.2 Vacuum Package

The vacuum package is the most frequently used package type for meat where a modified atmosphere is applied. The meat is put into a pouch made of a suitable plastic film, the air is evacuated, the pouch collapses around the product, and the pouch is sealed. Other technical procedures are also used, eg, a hot web can be molded to the meat on a stiff lower web by establishing a vacuum on both sides of the upper web, then the vacuum above the upper web is released (vacuum skin package).

However it is produced, a vacuum packed product is encircled by a plastic film that ideally should be gas impermeable. There is no vacuum inside these packages. The air space is strongly restricted, and the proportions of oxygen and carbon dioxide are altered by the scavenging of oxygen by the meat and the microflora, and by the production of carbon dioxide. These changes affect the metabolism of the microflora (growth and end-product formation), and the product's shelf life will be prolonged.

The evolving carbon dioxide in a meat package can originate from the following: (1) diffusion from a preformed pool in the meat, (2) aerobic energy metabolism of meat cells, (3) other biochemical reactions of the meat cells, and (4) microbial activity.[44] It is not clear which of these are the most important. However, the presence of oxygen seems to be of minor importance for the initial carbon dioxide production, when about 2×10^{-3} ml carbon dioxide cm^{-2} of meat surface per hour is produced.[44]

Provided the vacuum package is gas tight, the partial pressure of carbon dioxide ideally will gradually approach 1 atm (101 kPa) and the partial pressure of oxygen will decrease below normal detection levels (<0.0001 atm = <10 kPa). In heat-treated meat, however, the rate of production of carbon dioxide and oxygen scavenging decrease. The change of the gas phase is, in this case, mostly due to microbiological action alone.

Because carbon dioxide is highly soluble in water, even after a considerable amount has been produced, the appearance of the package can remain largely unaffected. In vacuum packaged fresh meat that has been chill stored for some days, the concentration of carbon dioxide will be expected to be least 20% to 40% (v/v),[138] and frequently it is around 90% (v/v), measured in the tiny head space of the package,[112] and the oxygen concentration will be below 1% (v/v).[112,138] The concentrations vary and are dependent on several factors, eg, the quality of the package film, head space, storage temperature, meat quality, and the composition and activity of the microflora.[62] From the bacteriological point of view, this is the major drawback with the vacuum packaging of meat, ie, even if a satisfactory shelf life is achieved in a majority of the packages, there will always be a certain number with an unsuspected short shelf life, due to leakage and/or unsatisfactory carbon dioxide generation. As a consequence, the safety margin between maximum obtainable shelf life and the storage time must be kept wide.

In general, vacuum packaging of refrigerated meat can extend the shelf life three to five times compared with that obtained in air.[62,112] However, for meat with a high pH, for example DFD beef (dark firm dehydrated beef) or poultry, the extension of shelf life is smaller,[26,46,107] and is only about twice that obtained in air.[62]

Vacuum packaging is applied mostly for primal cuts in wholesale storage, and only occasionally for display packs, most frequently for poultry. When discussing vacuum packaging, the relative importance of carbon dioxide inhibition in relation to oxygen limitation is important. It is easy to jump to the conclusion that oxygen is the key factor, just because the product normally is spoiled by aerobes.[119] However, with respect to *Pseudomonas* and meat, the following facts must be held in mind: (1) species of *Pseudomonas* are sensitive to carbon dioxide and their growth is significantly retarded by only a few percent of carbon dioxide in the gas phase (Figure 10-2); (2) the meat packages are stored at refrigeration temperatures where the gas solubility is high; and (3) *Pseudomonas* has a high affinity for oxygen, ie, the organism can grow at relatively low partial pressures of oxygen.[41,107,109] Ingram[82] concluded that "a large, even major, part of the inhibition in an impermeable wrap might be due *not* to lack of oxygen but to an increased concentration of carbon dioxide" (see Figures 10-4 and 10-7).

Conversely, the oxygen content is far from unimportant. Under atmospheres with an increased partial pressure of carbon dioxide, where bacteria sensitive to carbon dioxide have been inhibited, the oxygen content will affect the composition of the

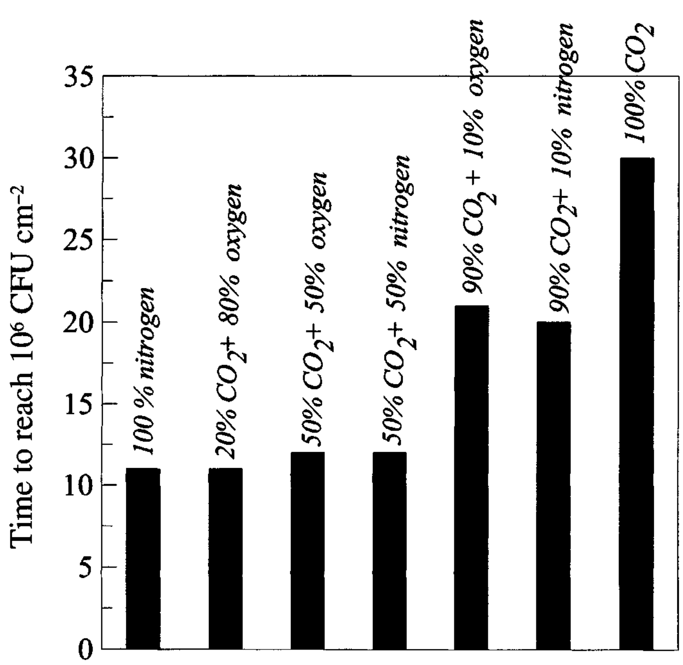

Figure 10-4 The Effect of Initial Gas Atmosphere on the Time To Reach an Aerobic Count of 10^6 Cell Forming Units (CFU) cm^{-2} on Pork (with bones) Stored at 4°C. The pork had an initial bacterial load of about 10^2 CFU cm^{-2} and was packed in gas-tight, aluminum-laminated pouches with an initial head space of approximately twice the volume of the product. *Source:* Copyright © 1989, Göran Molin.

secondary flora (Tables 10-1 and 10-2). Furthermore, oxygen can also affect end-product formation in a negative direction (ie, production of compounds with adverse effects on product quality).[11,149]

10.4.3 Packing in Modified Atmosphere with a Head Space

Packages designed with a head space containing a modified composition can be used for both display and bulk packaging. The proportions of oxygen and carbon dioxide can be selected either (1) to obtain a limited shelf life of meat and retain a desirable color or (2) for a maximal prolongation of shelf life without any major considerations about the meat color. The former is mostly used for display packaging and the latter for bulk packaging.

The color of raw meat is to a high degree dependent on the oxidation state of myoglobin.[59] The biological role of myoglobin is to carry oxygen, and the oxygenated myoglobin (oxymyoglobin) is bright red. This is the color most commonly associated with freshness because the consumer is accustomed to the appearance of meat exposed to the oxygen of the air. The oxygenation is rapidly reversible with

Table 10-1 Taxa Dominating the Aerobic Count of Pork Loins Stored at 4°C in Different Initial Gas Atmospheres, When the Count Had Reached the Level of about 10^6 CFU cm^{-2} of Meat Surface

Gas Atmosphere [% (v/v)]	*Dominating Taxa*
air	*Pseudomonas*
100% nitrogen	*Enterobacteriaceae*
20% CO_2 + 80% nitrogen	*Enterobacteriaceae* + *Aeromonas*
20% CO_2 + 80% oxygen	*Brochothrix thermosphacta*
50% CO_2 + 50% nitrogen	*Enterobacteriaceae* + *Brochothrix thermosphacta*
50% CO_2 + 50% oxygen	*Brochothrix thermosphacta*
90% CO_2 + 10% nitrogen	lactobacilli*
90% CO_2 + 10% oxygen	lactobacilli* + *Brochothrix thermosphacta*
100% CO_2	lactobacilli*

Note: The time required to reach 10^6 CFU cm^{-2} of meat surface is shown in Figure 10-4.[112]

*Mainly taxa within the genus *Lactobacillus* of the phenotypes described by Shaw and Harding[137] and Borch and Molin,[12] but the count may also include taxa of the genera *Leuconostoc* and *Carnobacterium*.[12,23,137] *Carnobacterium* has, from the taxonomic perspective, no close relationship with *Lactobacillus* and *Leuconostoc*.[21]

Source: Copyright © 1989, Göran Molin.

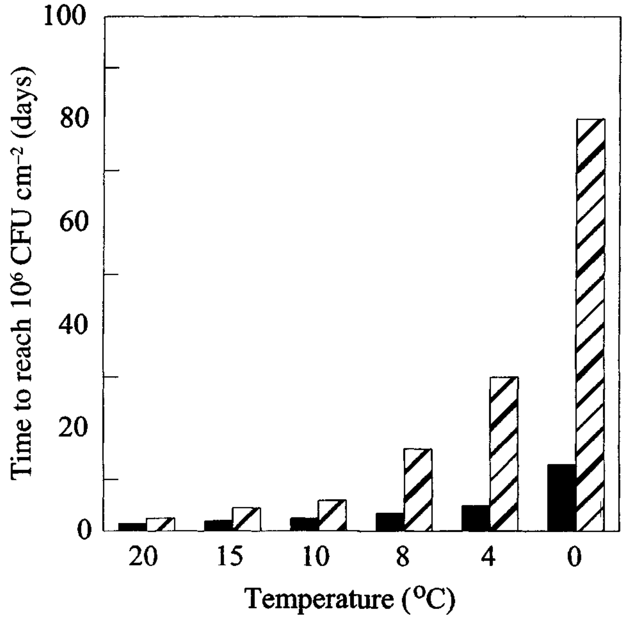

Figure 10-5 The Effect of Temperature and Carbon Dioxide on the Time Required for the Aerobic Count on Pork Loins To Reach to 10^6 CFU cm^{-2} on the Meat Surface. The head space at the time of packaging was equal to four times the volume of the product.[112] Initial head space air (filled columns), initial head space 100% carbon dioxide (hatched columns). *Source:* Copyright © 1989, Göran Molin.

changes in the partial pressure of oxygen; at low partial pressures the meat color darkens due to the purple color of the deoxygenated myoglobin. The time taken for myoglobin to turn to oxymyoglobin when exposed to air is 0.5 to 1 hour.[136] However, this change takes longer if the meat has been exposed to high concentrations of carbon dioxide (4 to 5 hours).[135]

On the other hand, myoglobin can also be oxidized, particularly at reduced partial pressures of oxygen, and can form metmyoglobin, ie, in the active site of the myoglobin molecule Fe^{2+} is oxidized to Fe^{3+} and the pigment turns brown.[59] This oxidation is only slowly reversible by enzyme-mediated metmyoglobin reduction, and the reduction capacity is limited and decays during storage.[50,59] Thus, in air-stored meat the color change due to metmyoglobin to some degree reflects lack of freshness of the meat. Deoxygenated myoglobin is more susceptible to oxidation than oxymyoglobin, thus an atmosphere with a low partial pressure of oxygen not only changes the meat color due to the color of the deoxygenated myoglobin, it also enhances the formation of metmyoglobin.[50]

If the color is important, as in a display package, the meat can be packed in an atmosphere of 70% to 80% (v/v) oxygen (to ensure a fresh red color) and 20% to 30% carbon dioxide in order to obtain a reasonable shelf life. This type of package gives a shorter shelf life than vacuum packaging[23,52,138,149]; but provides a shelf life about twice that of storage in air.[138]

To ensure a long shelf life the modified atmosphere should provide as high a partial pressure of carbon dioxide as feasible. As seen in Figure 10-4, (1) the slowest bacterial multiplication was obtained in 100% carbon dioxide, (2) the differences between growth rate in 20% (v/v) carbon dioxide and in 50% carbon dioxide is relatively small, and (3) it is of little consequence for the increase in the aerobic count whether or not an atmosphere of 50% carbon dioxide (or more) is supplemented with oxygen or nitrogen.

Metmyoglobin formation increases in meat stored in a modified atmosphere with a high amount of carbon dioxide for at least two reasons: (1) a reduced partial pressure of oxygen increases metmyoglobin formation; the formation is optimal at 0.1–1.3 kPa in a chemically defined system,[58] and optimal between 0.7–2.7 kPa for beef stored at 0°C[95]; and (2) the shelf life is extended and so, therefore, is the time available for metmyoglobin formation.

In bulk packs of meat, the color might be of lesser importance and the composition of the gas phase can be chosen for purely bacteriological reasons. In this case, the best results are obtained with pure carbon dioxide (Figure 10-4). The question of the color does not apply in poultry and is less pronounced in pork than in beef, therefore, for these products 100% carbon dioxide is preferable. Often there are problems in achieving a sufficiently long shelf life for fresh poultry.[107]

The high water solubility of carbon dioxide may sometimes be a problem in some modified atmosphere packages where it may lead to such a decrease in gas volume as to cause collapse of the package. This can be avoided either by incubating the product in the specific gas atmosphere prior to packing or by including nitrogen in the gas mixture. However, the concentration of carbon dioxide in the gas phase should, for microbiological reasons, initially be at least 70% (v/v).

The composition of the atmosphere in a package will change during storage, not only due to the carbon dioxide solubility, but also due to biological activity of both the meat and the microflora. The magnitude of such changes will be greatest in packs with a low ratio of the head space to meat volume. In practice the head space should be at least twice that of the product volume to ensure a reasonably stable composition of the gas atmosphere.[62]

If meat, eg, pork loins, is stored at a temperature close to the freezing point and in pure carbon dioxide, a shelf life of several months can be achieved (Figure 10-5).[9]

10.4.4 Microbial Flora

Figures 10-4 and 10-5 do not reveal whether different gas mixtures influenced the composition of the bacterial flora. The proportions of carbon dioxide and oxygen not only influence the rates of increase of the total numbers of bacteria, but also control the types of bacteria that dominate the flora and that presumably spoil the meat.[23] The dominant groups of bacteria when the "aerobic count" reaches about 10^6 CFU cm^{-2} of meat surface of the pork loins, shown in Figure 10-4, are indicated in Table 10-1. Similar results have been shown by others.[9,45,46,106,122]

The microflora in vacuum packages mimic the situation in modified atmosphere packages; poor packages with low carbon dioxide concentrations result in spoilage by enterobacteriaceae and *Aeromonas* while lactobacilli dominate the microflora of effective vacuum packages. However, a vacuum package is unsuitable for the storage of high-pH beef. The meat in these packages is often spoiled by enterobacteriaceae and *Aeromonas*, which is able to produce hydrogen sulfide[25,26] and some lactobacilli that under these conditions are able to form hydrogen sulfide.[37] The changes in the composition of the flora influence the eating quality of the meat and, hence, the shelf life. From a microbiological point of view, the optimal gas atmosphere for refrigerated meat is pure carbon dioxide, but after storage for a prolonged period in 100% carbon dioxide, the surface of the meat may acquire a porous gel structure.[62]

10.5 THE USE OF MODIFIED ATMOSPHERES IN PRESERVATION OF FRESH FISH

10.5.1 Spoilage

The microbial flora of fresh fish is heterogeneous, and, for example, includes the following: *Acinetobacter*, coryneforms, *Cytophaga, Flavobacterium, Micrococcus, Psychrobacter* (former "*Moraxella*-like"), *Aeromonas, Shewanella,* and *Pseudomonas*.[79,113,114,146,154] The initial numbers of bacteria on fish are normally higher than on mammalian meat. The living fish harbors bacteria on the skin (10^3–10^5 cm^{-2}),[79] gills (10^3–10^4 g^{-1} [79]) and in the intestines (up to 10^9 g^{-1} [79]), and even after filleting the count is often close to 10^5 CFU cm^{-2}.[109,113,146,154]

The high initial numbers of bacteria shorten the shelf life and result in a more complex bacterial flora after storage. The pH of fish tissue is usually relatively high (6.2–6.5), allowing rapid bacteriological spoilage. In spite of this, the microflora reaching high numbers in a particular batch of fish will be relatively homogeneous.[114]

The dominant groups of bacteria on fish stored under refrigeration are *Pseudomonas* (mostly *Pseudomonas fragi* and *Pseudomonas fluorescens*) and *Shewanella putrefaciens*, and to a lesser extent *Aeromonas* and *Psychrobacter*[79,145] (Figure 10-6).

Fish from warmer waters often harbor a higher proportion of Gram-positive bacteria[79] than those from colder waters and, due to the lower numbers of psychrotrophic bacteria, the shelf life at refrigeration temperatures is generally longer.[16,140] The similarities in the spoilage flora of fish and of mammalian meat are obvious but there is also, at least, one striking difference, ie, a substantial part of the fish flora consists of *Shewanella putrefaciens*.[145] This causes a problem because *S. putrefaciens* produces a spec-

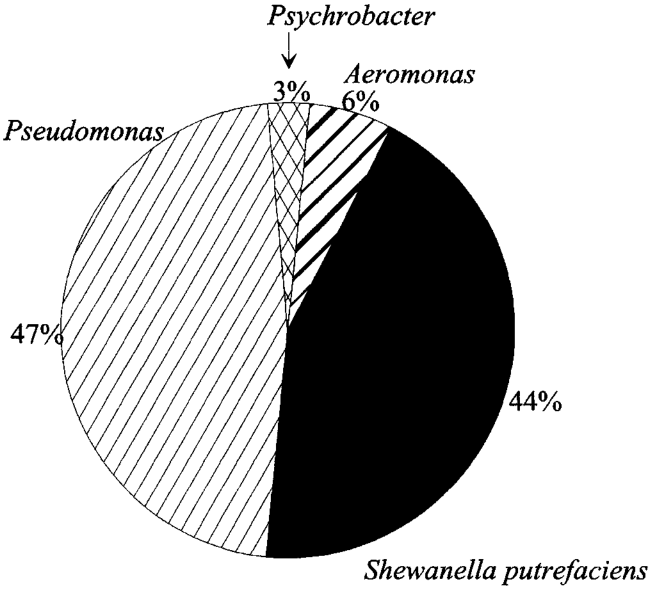

Figure 10-6 The Distribution of Different Bacterial Taxa Dominating the Flora of Swedish Fish Fillets When the Aerobic Count Approached 10^8 CFU g^{-1} after Storage in Air. The fillets had been stored at 2° to 8°C and represented a wide variety of different fish species.[145] The *Aeromonas* were all isolated from fish originating from Swedish inland waters.

trum of end-products during growth that are extremely deleterious to eating quality. Some of these can also be produced by *Pseudomonas*, for example, but the deleterious abilities are especially pronounced for *S. putrefaciens*, which produces hydrogen sulfide and also reduces trimethyl-amine-oxide, present in most marine fish, to trimethyl-amine[93,96]; the latter provides the product with an offensive spoiling fishy smell and the former with a putrid odor.[79] A factor causing problems with packaging in modified atmospheres is that *S. putrefaciens*, compared with *Pseudomonas* and enterobacteriaceae, is relatively resistant to carbon dioxide.[113,154] Occasionally *S. putrefaciens* also causes problems on mammalian meat; a typical situation is spoilage due to hydrogen sulfide formation in vacuum packed beef of high pH.[63] pH is a powerful controlling factor for the microflora at low temperatures; *S. putrefaciens* and *Aeromonas* particularly are less competitive at pH below 6.[23,26,61]

Furthermore, *Shewanella putrefaciens* is a heterogeneous species and the typical strains found growing on fish comprise at least two distinct groups. Both differ considerably from the type strain of *Shewanella putrefaciens*.[145,151] Clinical isolates designated as *S. putrefaciens* seem to a high degree to be misidentified and are, instead, *Shewanella alga*.[122] *Photobacterium phosphoreum*-like bacteria may grow and cause problems on fish stored in modified atmosphere.[27]

10.5.2 Modified Atmosphere

The general effect of carbon dioxide on the increase of the aerobic count of fish (Figure 10-7) is similar to that on mammalian meat (Figure 10-4). The increase in numbers was similar whether or not the carbon dioxide was supplemented with oxygen or nitrogen. The effect of the gas atmosphere on the proportion of *Shewanella putrefaciens* is of special interest because of the deleterious effect of this bacterium. The effect of the initial composition of the gas phase on the proportions of *S. putrefaciens* and lactobacilli in the aerobic count on fish is shown in Table 10-2.

Increased concentrations of carbon dioxide reduced the proportion of *S. putrefaciens* in the flora, but the effect was much less than that on *Pseudomonas*. The proportion of *S. putrefaciens* was lowest in either pure carbon dioxide or 10% (v/v) oxygen in 90% carbon dioxide (Table 10-2), but the growth-retarding effect was greater in 100% carbon dioxide (Figure 10-7). Nevertheless, from the microbial point of view, it is better to mix carbon dioxide with oxygen than with nitrogen when it comes to packaging fresh fish in modified atmosphere. The apparent stimulating effect of oxygen on the proportion of lactobacilli in the flora (and the coincident retarding effect on the proportion of *S. putrefaciens*) might be due to the ability of many lactobacilli strains to produce H_2O_2 in the presence of oxygen.[13]

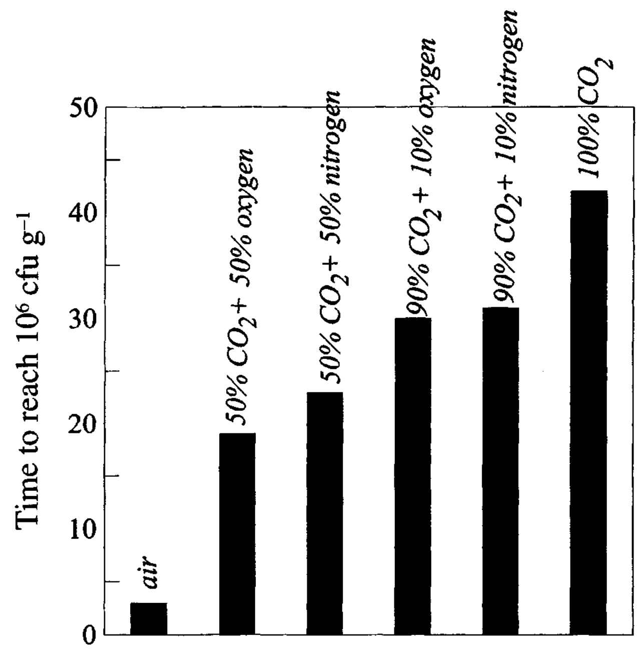

Figure 10-7 The Effect of Initial Gas Atmosphere on the Time To Reach an Aerobic Count of 10^6 Cell Forming Units (CFU) g^{-1} of Cod Fillet Stored under Atmospheric Pressure and at 2°C. Fillets were enclosed in glass jars and the head space was equal to six times the volume of the product. *Source:* Reprinted with permission from I.M. Stenstrom, Microbial Flora of Cod Fillets Stored at 2°C in Different Mixtures of Carbon Dioxide and Nitrogen/Oxygen, *Journal of Food Protection*, Vol. 48, pp. 585–589, © 1985, International Association of Milk, Food and Environmental Sanitarians, Inc.

10.5.3 Temperature

In comparison with mammalian meat, the shelf life–prolonging effect of packaging fish in modified atmosphere is less pronounced. This is due probably to the presence of *S. putrefaciens* on the fish (Table 10-2). It is particularly important to combine the modified atmosphere with a low refrigeration temperature. This is shown for herring fillets in Figure 10-8, and similar results have been presented by others.[31,129,142]

Table 10-2 The Effect of Initial Gas Atmosphere on the Proportion of *Shewanella putrefaciens* and Lactobacilli of the Aerobic Count of Cod Fillets Stored at 2°C, When the Count Had Reached about 10^6 CFU g^{-1} of Fish Tissue

Gas Atmosphere [% (v/v)]	S. putrefaciens *(% of the flora)*	*Lactobacilli (% of the flora)*
air*	62	0†
50% CO_2 + 50% O_2‡	38	44
50% CO_2 + 50% N_2§	75	3
90% CO_2 + 10% O_2‖	12	85
90% CO_2 + 10% N_2¶	30	62
100% CO_2#	12	80

Note: The time required to reach 10^6 CFU g^{-1} of fish tissue is shown in Figure 10-7.[146]

*Additional dominating taxa of the flora were *Pseudomonas*, 33%, and *Psychrobacter*, 5%.

†No isolates were identified as lactobacilli.

‡Additional dominating taxa were *Brochothrix thermosphacta*, 10%; *Pseudomonas*, 3%; *Psychrobacter*, 3%; *Vibrionaceae*, 3%.

§Additional dominating taxa were *Enterobacteriaceae*, 12%; *Vibrionaceae*, 5%; and *Brochothrix thermosphacta*, 3%.

‖Additional dominating taxum was 3% *Pseudomonas*.

¶Additional dominating taxum was *Brochothrix thermosphacta*, 8%.

#Additional dominating taxa were *Vibrionaceae*, 3%; *Brochothrix thermosphacta*, 3%; *Micrococcus*, 3%.

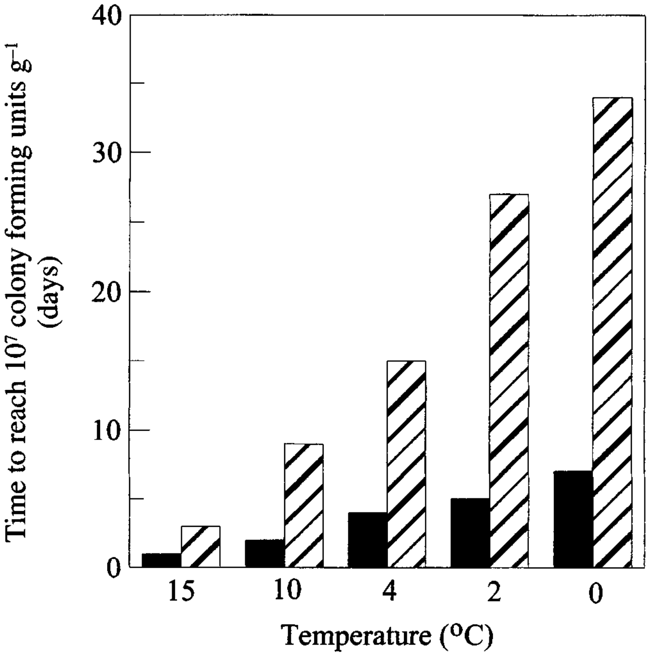

Figure 10-8 The Effect of Temperature and Carbon Dioxide on the Time Required for the Aerobic Count on Herring Fillets To Reach to 10^7 Cell Forming Units (CFU) g^{-1}. Fillets were enclosed in glass jars and the head space was equal to ten times the volume of the product. Initial head space air (filled columns) or 100% carbon dioxide (hatched columns). *Source:* Reprinted with permission from G. Molin and I.M. Stenstrom, Effect of Temperature on the Microbial Flora of Herring Fillets Stored in Air or Carbon Dioxide, *Journal of Applied Bacteriology*, Vol. 56, pp. 275–282, © 1984, Blackwell Science, Ltd.

Although at relatively high refrigeration temperatures, the retarding effect of pure carbon dioxide is clear (Figure 10-8), the controlling effect on the microflora is much less at higher temperatures, ie, the proportion of *S. putrefaciens* is high and true shelf life is considerably shorter than that depicted by the aerobic count (Table 10-3). The proportion of *S. putrefaciens* decreases with increasing concentration of carbon dioxide (Table 10-2) and decreasing temperature (Table 10-3). Thus, it is clear that *S. putrefaciens* is relatively resistant to carbon dioxide but is inhibited by a combination of low temperature and high carbon dioxide concentration.

10.5.4 Practical Considerations

The bacterial load of fish depends on the freshness of the product. The time to reach a certain number of bacteria does not necessarily determine the true shelf life of the product. This depends on odor and flavor of the fish and is often shorter than the shelf life indicated by the bacterial numbers.[27,75] Nevertheless, the shelf life can be extended by increased levels of carbon dioxide.[31,101,129,130,142]

Shelf life, of course, varies between different fish species and depends on how the fish is handled from the time of catching.[79] Using Norwegian cod as an example, the shelf life in air for this product is normally about 8 days at 0°C.[144] The following are crucial for the quality and shelf life: (1) the cod is gutted and deblooded as quickly as possible, and (2) it is rapidly cooled to 0°C.[144] A system based on modified atmosphere in consumer packs was developed for this product, and is shown schematically in Figure 10-9.[144]

Before packaging, the fillets were dipped in 20% (w/v) chilled salt (NaCl) solution for 12–14 seconds (Figure 10-9). The dipping resulted in a salt concentration of about 1% in the fillets (but no apparent salty taste). The salt dip reduced the drip in the package and also cooled down the fillets before packaging. The modified atmosphere package makes cooling difficult. The system provided a shelf life of the cod of 14 days from catching to consumption.[144] The cod fillets (250–350 g) were packed in an atmosphere of 50% (v/v) CO_2 and 50% O_2 in a flexible package with a volume of 750 cm^3 (package machine, Multivac R 7000; laminate, PVC/PE).[144] It is recommended that the ratio of product to package volume be 40% or be-

Table 10-3 The Proportion of *Shewanella putrefaciens* and Lactobacilli of the Aerobic Count of Herring Fillets Stored at Different Temperatures, in an Initial Atmosphere of Pure Carbon Dioxide, When the Count Had Reached about 10^7 CFU g^{-1} of Fish Tissue

Temperature (°C)	S. putrefaciens *(% of the flora)*	*Lactobacilli (% of the flora)*
10*	49	25
4	97	3
2	0†	100‡
0	0†	100‡

Note: The time required to reach 10^7 CFU g^{-1} of fish tissue is shown in Figure 10-8.[113]

*Additional dominating taxa were unidentified Gram-positive rods, 13%; *Enterobacteriaceae*, 5%; *Acinetobacter calcoaceticus*, 3%; *Flavobacterium*, 3%; *Cytophaga-Flexibacter* group, 3%.

†No isolates were identified as *Shewanella putrefaciens*.

‡All identified isolates were *Lactobacillus*.

low. A higher concentration of carbon dioxide was not used because the package would deflate due to the water solubility of the carbon dioxide.

10.6 THE USE OF MODIFIED ATMOSPHERES IN PRESERVATION OF PROCESSED MEATS

10.6.1 General

The expression "processed meats" includes a wide variety of different products, not always within stringent borderlines. The group includes traditional cured meats such as bacons, sausages, and hams, and also convenience food products such as premade hamburgers and meatballs. Especially important factors to consider are whether the product has been heat treated, the concentration of NaCl, and the water activity. The products that provide different environments for spoilage microorganisms and rely on different preservation systems will not be discussed in detail in this section. Instead, some general principles that have relevance for most of the products will be discussed. However, there can be special conditions for specific products that can overrule some of the general principles.

10.6.2 Raw Cured Meats

The storage of raw, cured meats in modified atmospheres differs from that of fresh meat primarily because of the controlling effect of NaCl on the spoilage flora. Thus, at low temperatures, about 3% NaCl will reduce both the growth rate of the spoilage flora

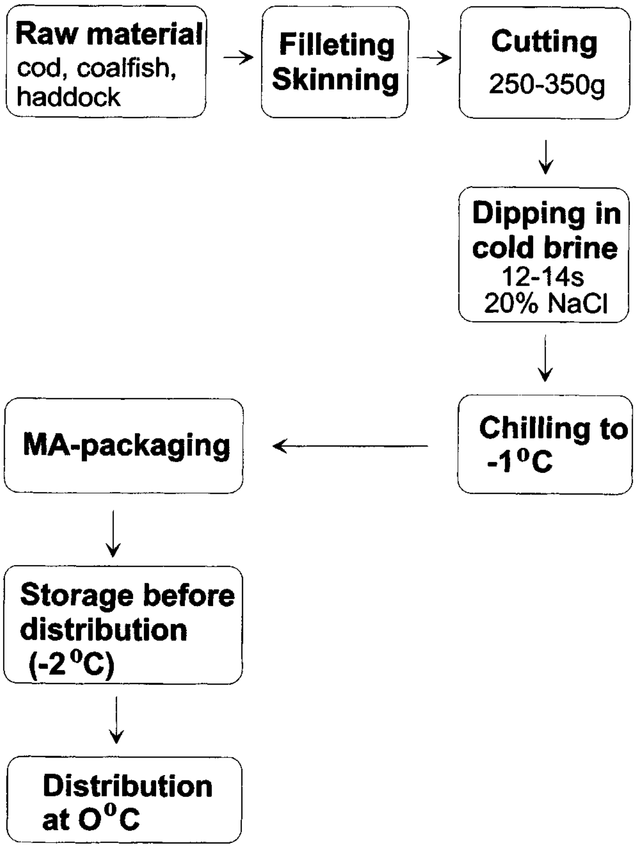

Figure 10-9 A System for Production of Retail Packs of Norwegian Cod Fillets in Modified Atmosphere (50% CO_2 and 50% O_2)

and the proportion of *Pseudomonas* with respect to *Brochothrix thermosphacta*.[9] This effect will be in addition to that of the modified atmosphere, and long shelf lives can be obtained with the combination of low temperature, high carbon dioxide concentrations, and NaCl (3% NaCl).[9] The flora under these conditions is dominated by lactobacilli.[9] If additional growth-restricting factors are added to that of NaCl, or if the NaCl concentration is increased, for example, to decrease the water activity to 0.94, the effect of carbon dioxide on the increase in "aerobic count" will diminish.[7] This is because the environmental selection pressure on the spoilage flora is optimal for lactobacilli, and the growth rate for this group is close to its minimum. If the environment is made even more hostile, the growth of lactobacilli will stop completely.[7] If these types of product are stored in air, they will be overgrown by yeasts, and if the water activity is decreased further, by molds.[7,112] Molds are easily eliminated by storage in strictly oxygen-limited atmospheres and yeast growth may be substantially reduced.[7,112]

10.6.3 Heated Cured Meats

Heated cured meats such as frankfurter sausage or smoked pork loin differ from fresh meat or fish in the following respects:

1. The mammalian cells do not metabolize and neither consume any oxygen nor produce any carbon dioxide. Only the microflora consumes oxygen and releases carbon dioxide. These activities proceed slower in a package of processed meat than in packages of fresh meat or fish.[10]
2. The processing has changed the microbiological flora, ie, taxa such as *Pseudomonas*, *enterobacteriaceae*, *Shewanella*, and *Aeromonas* have been eliminated, or greatly reduced in numbers by the heat process, and post-process contaminants of these groups have severe difficulties in growing; *Brochothrix* has difficulties in competing.[10,12,14] This leads to a prolongation of shelf life even in air, where the growing microflora mostly will be dominated by yeasts (or, if the water activity is low enough, by molds).[112] Thus, if the shelf life must be further extended, the products can be vacuum packed or gas packed.

Vacuum Packaging

By vacuum packaging of, for example, emulsion sausages, the growth of yeasts is reduced, or prevented, and the dominant microorganisms are homofermentative *Lactobacillus* species.[12,14] Also substantial numbers of *Leuconostoc* and *Carnobacterium* can be found.[12]

The effect of storage temperature on the increase of aerobic count in vacuum packed emulsion sausage is shown in Figure 10-10. The spoilage of sausages is not directly linked to the bacterial numbers.[91] The flavor can be acceptable long after the total count has reached its maximum,[14] but before the sausages take on an offensive odor or taste, they become unacceptable due to slime formation; no slime is visual before the total count has exceeded 10^6 CFU g^{-1}.[14,53]

Gas Package with a Head Space

A major drawback of vacuum packing is that the product gets a wet surface. This reduces the acceptability of the product but has also bacteriological implications, eg, the product is more likely to become slimy. A gas package with a substantial head space can provide the product with a dryer surface, and less drip (free meat juice in the package). However, there must be nitrogen in the gas because with pure carbon dioxide the product will still exude water.[10,14]

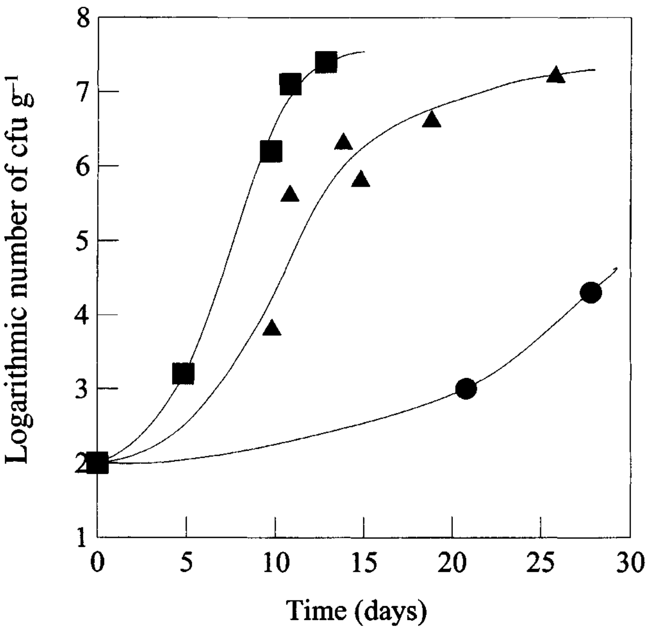

Figure 10-10 Increase in Aerobic Count on Vacuum Packed Emulsion Sausage Stored at Different Temperatures (Swedish Falukorv[12]). Filled squares indicate storage at 8°C, filled triangles indicate 4°C, and filled circles indicate 0°C. *Source:* Copyright © 1989, Göran Molin.

Packaging emulsion sausages in pure nitrogen resulted in a longer shelf life than packaging in pure carbon dioxide, ie, the time until the flavor deteriorated and slime was detected was longer in nitrogen than in pure carbon dioxide.[14] No advantages or disadvantages in respect to shelf life were seen with carbon dioxide concentrations up to 50% (v/v) in nitrogen.[14] However, the addition of a certain amount of carbon dioxide (20% to 40%) in the nitrogen can be a hurdle for yeast or mold development in packages where some oxygen is present by, for example, accidental leakage.

Oxygen should be avoided in packages for heated, cured meats for at least the two following reasons: (1) oxygen enables the growth of molds and encourages growth of yeasts and (2) oxygen helps many lactobacilli to produce more adverse end-products, eg, H_2O_2, acetic acid, formic acid, and acetoin/diacetyl.[11,14] Hydrogen peroxide causes green discoloration.[53]

The recommended gas mixture for packaging heated, cured meats in modified atmosphere is 20% to 40% (v/v) carbon dioxide in 60% to 80% nitrogen.

10.7 THE USE OF MODIFIED ATMOSPHERES IN PRESERVATION OF OTHER FOODS

10.7.1 Milk and Cheese

Raw milk is mainly spoiled by *Pseudomonas fluorescens* biovar 1, *Pseudomonas fragi, Pseudomonas lundensis,* and *Pseudomonas fluorescens* biovar 3.[152] If the milk was pasteurized, about half of the packed milk is spoiled under refrigeration by the same taxa of Gram-negative bacteria. The other half is slowly spoiled by *Bacillus* species.[152] The bacteriological shelf life of refrigerated milk can be prolonged by applying an increased partial pressure of carbon dioxide[92] (see Chapter 22).

In cheese, the manufacturing process has suppressed the spoilage flora of fresh milk, and normally the major threat is yeasts and molds on the surface. They can be suppressed by a modified atmosphere containing low oxygen levels and increased carbon dioxide concentrations.[48,103] Vacuum packaging occasionally fails to protect against molds[81] (see Chapter 22).

10.7.2 Bread

Bread can be packed in vacuum or modified atmosphere in order to delay staling[3] or to hinder the growth of molds,[100] eg, 70% to 80% (v/v) carbon dioxide and 20% to 30% nitrogen.

10.7.3 Fruits and Vegetables

The rate of respiration of fruits and vegetables can be reduced by raising the partial pressure of carbon dioxide and decreasing the partial pressure of oxygen. However, a too high level of carbon dioxide will result in tissue damage, similar to that caused by too low temperatures.

Several processes in vegetables and fruits other than respiration are influenced by the gas atmosphere, eg, ripening, production of ethylene, breakdown of chlorophyll, and structural changes due to the breakdown of pectin. These processes are different for different products, eg, between apples and pears.[56] Thus, the design of controlled or modified atmospheres is different for different types of vegetables and fruits.[120] Apples can be stored in a mixture of 5% to 10% (v/v) carbon dioxide and about 3% oxygen and nitrogen as the filler; tomatoes can be kept in an atmosphere of 3% to 9% carbon dioxide, 3% to 9% oxygen and nitrogen[55]; and a suitable atmosphere for strawberries is 10% to 20% carbon dioxide, with a low oxygen concentration and nitrogen as a filler.[17,71]

Storage of fruits and vegetables in modified atmospheres is done to retard the ripening of the product, and to reduce quality losses during storage. The latter includes microbiological spoilage by Gram-negative bacteria such as *Pseudomonas* and *Enterobacteriaceae*, and by fungi.[90] However, the carbon dioxide concentrations are relatively low and even if *Pseudomonas* and several fungi are relatively sensitive to carbon dioxide, the microbiological aspects of storage of vegetables and fruits in modified atmospheres are mostly of secondary importance.

10.8 SOME SAFETY ASPECTS OF MODIFIED ATMOSPHERES

10.8.1 *Clostridium botulinum*

Consideration of safety aspects of packaging foods in modified atmospheres started with the fear that vacuum packaging could increase the hazards of botulism, ie, that the anaerobic conditions in a vacuum package could facilitate growth and toxin formation by *Clostridium botulinum*.[28,57,82] Furthermore, it could be argued that vacuum packaging, or even more so, modified atmosphere packaging with a high partial pressure of carbon dioxide, increases the shelf life of a product to such an extent that there is more time available for growth and toxin production by *C. botulinum* to occur. These problems might be especially pronounced in connection with individual retail packages where the chance of temperature abuse is more pronounced than at the supplier level.[57] However, the fears for growth and toxin formation of *C. botulinum* in refrigerated foods packed in modified atmospheres seem to be exaggerated.

First, irrespective of the gas atmosphere surrounding the piece of meat or fish, the conditions are anaerobic enough for growth of *C. botulinum* a few mm below the tissue surface[5,82] or below a metabolizing biofilm of microorganisms.

Second, *C. botulinum* is not alone in these types of products. It has to compete with a spoilage flora that either will be dominated by taxa that severely affect the eating quality or by a dominating fraction of lactobacilli (see above), which are well known for their ability to suppress clostridia.[29,30,85]

Third, even though increased partial pressures of carbon dioxide have been shown to stimulate the germination of clostridia spores,[40,49] a modified atmosphere of 100% carbon dioxide suppressed toxin production by *C. botulinum* in comparison with 100% nitrogen.[34] No difference in growth rates of *Clostridium perfringens* were found between 100% carbon dioxide and 100% nitrogen,[126] while Mead[107] reported that the growth of *C. perfringens* was retarded by carbon dioxide.

In conclusion, the earlier presumed hazards for growth and toxin formation by clostridia in refrigerated meats and fish packed in modified atmospheres seem exaggerated. Most products are judged inedible well before any toxin of *C. botulinum* can be found in the products. This general conclusion was also drawn by others.[82,107]

10.8.2 Psychrotrophs with Pathogenic Potential

In the safety discussion concerning clostridia, it is important to remember that most clostridia are unable to grow at chill temperatures. Those able to do so, such as psychrotrophic strains of *Clostridium botulinum*, grow only poorly under these conditions, ie, they are unable to grow below 3°C.[131] However, there are also psychrotrophs with considerable pathogenic potential that can reach high numbers in refrigerated foods, for example, *Listeria monocytogenes*, *Aeromonas* species,[98,118,145] and *Yersinia enterocolitica*.[84,88,94] There might also be other species of the family enterobacteriaceae to consider, for example, *Citrobacter freundii* and *Enterobacter cloacae*.[97] Another potential threat, in refrigerated fish, might be *Vibrio* species.[98]

The Gram-negative bacteria occasionally occur as a significant part of the spoilage flora of refrigerated fresh meat (*Aeromonas* and enterobacteriaceae) and fish (*Aeromonas*, enterobacteriaceae, and *Vibrio*) stored in air or in modified atmospheres. Certain environmental parameters are beneficial for their development. For example, a high pH is beneficial for *Aeromonas*[124] and *Yersinia enterocolitica*[134] and a somewhat higher refrigeration temperature would be beneficial for the enterobacteriaceae.[8,33] Furthermore, a modified atmosphere with a relatively low partial pressure of carbon dioxide can favor the development of enterobacteriaceae and *Aeromonas*[33,66] (Tables 10-1 and 10-2). The best protection against *Aeromonas* and enterobacteriaceae is offered by the combination of low storage temperature and a modified atmosphere that provides a high partial pressure of carbon dioxide.[33]

10.8.3 *Listeria monocytogenes*

Listeria monocytogenes is not likely to compete successfully in refrigerated raw meat and fish.[47,66] Theoretically, a hazard may arise in modified atmosphere packs after extended storage periods, at moderate partial pressures of carbon dioxide, and in the presence of oxygen.[101,139,157] Another important parameter is the pH, ie, a pH below 5.0 will decrease substantially the likelihood for growth of *L. monocytogenes*.[47,69]

However, the problem with *L. monocytogenes* is more connected to products that are consumed directly without heat treatment and in products where the normal spoilage flora has been inhibited.[47] The modified atmosphere might eventually increase the hazards due to extended storage times. In general, a high partial pressure of carbon dioxide in combination with a low storage temperature will decrease the chances for growth of *L. monocytogenes*.[47,66] For example, the growth in cottage cheese is stopped by packaging in elevated concentrations of carbon dioxide.[19,48] Furthermore, the chances of *L. monocytogenes* growth decreases if the domination of lactobacilli in the product is strong.[47]

The possibility of growth of *L. monocytogenes* is relatively higher in, for example, some soft cheeses and in some vegetables,[47,121] and may be higher in preprepared salads packed in modified atmosphere with only a low concentration of carbon dioxide. Vegetables are seldom stored in atmospheres exceeding 10% (v/v) carbon dioxide. In the case of cheese, the chances of growth of *L. monocytogenes* can be decreased by packaging in modified atmospheres containing a high partial pressure of carbon dioxide.[19,48]

10.9 CONCLUDING REMARKS

Carbon dioxide is the most powerful gas component in modified atmosphere packaging to inhibit microbial growth. Growth inhibition increases with increase in the partial pressure of carbon dioxide and low temperature reinforces the growth-inhibitory effect.

The composition of the spoilage flora of refrigerated meat is controlled by the gas atmosphere according to the following principles:

1. *Pseudomonas*, which dominates the spoilage of meat stored in air, is retarded at a partial pressure of carbon dioxide of about 10 kPa (10% [v/v]) in the gas phase at atmospheric pressure).[54,109]
2. *Brochothrix*, enterobacteriaceae, and to some degree also *Aeromonas*, are the first to take over in atmospheres where *Pseudomonas* has been inhibited due to an increased carbon dioxide concentration[26,46,112] (Table 10-1).
3. *Brochothrix* is slightly more resistant to carbon dioxide than members of the enterobacteriaceae, but has a much slower growth rate under anaerobic conditions and is, because of this, outnumbered by enterobacteriaceae in an anaerobic environment. On the other hand, in the presence of oxygen *B. thermosphacta* will dominate due to its higher resistance to carbon dioxide.[23,26,46,112]

4. The growth of both enterobacteriaceae and *Brochothrix* is inhibited by partial pressures of carbon dioxide close to atmospheric pressure. The bacterial flora will instead be dominated by lactobacilli, primarily of the genus *Lactobacillus* but also substantial numbers of *Leuconostoc* and *Carnobacterium* can occur.[12,23,137]

Meat in modified atmosphere should preferably be stored at temperatures below 4°C.

One obstacle with modified atmosphere packaging of fresh fish is the growth of the relatively carbon dioxide–resistant *Shewanella putrefaciens*. The distribution of fresh fish, from the microbiological point of view, preferably ought to be performed at 0°C or below, and in a gas atmosphere of pure or nearly pure carbon dioxide. However, there can be considerations other than the microbiological ones, eg, the appearance of the product and the package, and the loss of water from the product. If the carbon dioxide is supplemented with another gas for reasons other than microbiological ones, it should be with oxygen. Nitrogen should be avoided in connection with fresh fish.

The partial pressure of carbon dioxide in modified atmosphere packaging of processed meat is less critical than for fresh meat. The growth of Gram-negative bacteria is retarded by factors such as NaCl and decreased water activity. The assignment of the modified atmosphere is to maintain a dry surface and keep out the oxygen; a suitable atmosphere is 20% to 40% carbon dioxide in 60% to 80% nitrogen. To ensure the absence of oxygen, an oxygen absorber can be included in the package.

The use of modified atmosphere packaging is increasing, and the packing technique and the control of the atmosphere within the package will most probably further improve in the future. Besides the effect on eating quality, it is equally important to consider the safety aspects of extended storage times. The longer the storage time, the greater the potential microbiological hazards. Sophisticated packaging systems for susceptible refrigerated foods must be combined with advanced systems for microbial control, founded on solid knowledge of microbial ecology of foods.

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11

The Effect of Redox Potential

J. Gareth Morris

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References

11.1 INTRODUCTION: OXIDATION-REDUCTION REACTIONS AND REDOX POTENTIALS

11.1.1 Background Theory

Oxidation of any substance (a *reductant)* is accomplished by loss of electrons. Thus, for example, when a Fe(II) ion is oxidized, it sheds an electron and is thus converted into a Fe(III) ion as follows:

$$Fe^{2+} \rightarrow Fe^{3+} + e^-$$

Yet for such an oxidation to occur, the electrons that are dispensed with must be accepted by some other substance, which thereby serves as an *oxidant.* Thus, the Fe(II) ion may have been oxidized by oxygen, which was in turn reduced to water:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

The overall process is a balanced oxidation-reduction reaction, represented as

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

Every oxidation-reduction may therefore be viewed as the consequence of the necessary interaction of an electron-donating and an electron-accepting "half reaction." The electron-donating component constitutes one *redox couple* and the electron-accepting component, another. Ideally, every redox couple is fully reversible, so that all can be represented by the following general equation:

$$a(\mathrm{Ox}) + b(\mathrm{H}^+) + ne^- \rightleftharpoons c(\mathrm{Red})$$

where Ox represents the oxidant and Red is the reductant formed from it by the acceptance of electrons (and sometimes of protons also).

Of the two redox couples that participate in an oxidation-reduction reaction, it follows that one must be the more eager to accept electrons. It is therefore possible on this basis to construct a relative scale of *redox potentials* of these couples, the redox potential being a measure of the *intensity* of its tendency to accept electrons.[57] As with any such scale, it is first necessary to define a set zero value. This is decreed to be the redox potential of a standard hydrogen redox couple (also known as the standard hydrogen electrode or standard hydrogen half-cell), which consists of hydrogen gas at 1 atmosphere pressure in equilibrium with an aqueous solution of hydrogen ions at unit activity (ie, pH 0). Were a redox couple of unknown potential to be made one half-cell in an electrochemical cell in which this standard hydrogen electrode (SHE) formed the second half-cell, then the maximum electromotive force in volts displayed by this cell (ie, when no current was allowed to flow) would be a measure of the potential difference between the redox couple and the reference zero. This value will be the redox potential (E_h) of the redox couple at the prevailing temperature and pH. Couples displaying positive values of E_h are more oxidizing than the SHE in proportion to the magnitude of the voltage difference. Similarly, couples more reducing than the SHE will have negative redox potentials; the more negative, the more reducing is the couple.

How redox potentials are measured in systems of bacteriological and biochemical interest has been fully described,[3,4,28,34,36,38,41,61] and methods of measurement of redox potentials in meat[71] and wine[81] have recently been published. In brief, a highly polished platinum electrode, when introduced into the system, registers its redox potential and establishes a measured electromotive force (potential difference) when it is then suitably connected to a stable reference electrode. In practice, it would be rather inconvenient to attempt to employ a standard hydrogen electrode for this reference purpose, so one of several commercially available electrodes is used in its place.[34] The potentials of these standard electrodes, relative to the hydrogen electrode, are known over a range of temperatures. Thus, for example, if a reducing biological redox couple registered at 30°C a potential difference ΔE of 562 mV against a saturated calomel electrode (whose potential at 30°C is +242 mV), then the redox potential of the test couple on the hydrogen scale (ie, its E_h value at 30°C) is –320 mV for

$$\Delta E_h = E_h \text{ (oxidizing couple)} - E_h \text{ (reducing couple)}$$
$$562 = +242 - E_h \text{ test couple}$$
$$\text{where, } E_h \text{ test couple} = 242 - 562 = -320 \text{ mV.}$$

Alternatively, the redox potential of the test couple versus the saturated calomel electrode could be recorded as an E_{cal} value of –562 mV, in which case its redox potential on the hydrogen scale would be readily calculable as (–562 +242) mV = –320 mV.

An important consequence of the ability to measure the maximum electromotive force (ΔE_h in volts) that would be developed by the conjunction of two redox couples is that it is thereby possible to calculate the electrical work that would be performed by the oxidation-reduction reaction were it to operate thermodynamically reversibly and isothermally. This work equals $nF\Delta E_h$ J mol^{-1}, where n is the number of electrons "lost" per molecule of reducing agent oxidized (or the quantity of electricity flowing through the cell measured in Faradays), and F is the Faraday constant, which equals 92,487 J V^{-1} mol^{-1}. Because this work represents the loss in Gibbs free energy of the system per mol of reductant oxidized, then

$$-\Delta G = nF\Delta E_h \text{ J mol}^{-1}$$

This is an important relationship in that not only does it form the basis of a very practical way of deriving values of ΔG for oxidation-reduction reactions, but, by highlighting the proportionality between the degree of exergony (spontaneity of reaction) and the span of E_h difference between the electron-donating and electron-accepting couples, it justifies the attention that is paid to the assignment of E_h values to such redox couples.

11.1.2 Nature of the Redox Potential of a Redox Couple

Because the essence of oxidation-reduction is the transfer of electrons between participant couples, an analogy may be drawn with acid-base interactions with their transfer of protons. It is a small step thereafter to conceive of *electron activity* as the analogue of proton activity and of *pe* values (negative logarithm of electron activity) as the redox counterpart of pH values. Indeed, in soil science and agronomy, much use has been made of such *pe* values.[6] Yet even those that use the term concede that, under conditions relevant to biological systems, electrons do not exist as free entities in any phase (except in metals). Thus to conceive of electron activity in the same terms as proton activity is not only misleading but is actually wrong. It is also unnecessary, since the E_h values of redox couples, when reported together with the conditions (temperature and pH) under which they were measured, give all the information that is required without having to invoke the complication of proxy *pe* numbers. On the other hand, it is legitimate for thermodynamic purposes to define the electrochemical potential of the electron in terms of the electrochemical potentials of the oxidized and reduced species of the redox couple.[83] In particular, the standard redox potential of a couple ($E°$) is an expression of the standard chemical potential of its electron(s). Although in thermodynamic terms, $E°$ is the redox potential of the couple in its standard state (unit activities of participating species at 25°C, 1 atmosphere pressure [atm]) it can be shown that it is also the redox potential displayed by the redox couple when its oxidant and reductant components are present at equal concentration (activity), whatever this happens to be in absolute terms. It is for this reason that the symbol E_m (for midpoint potential) is frequently used in place of $E°$ to represent the standard redox potential of a redox couple in its 50% reduced condition (Figure 11-1). This midpoint potential is the standard potential about which the actual potential (E_h) can vary in proportion to the extent to which the ratio of oxidant to reductant differs from unity. The relationship is expressed in the following Nernst equation:

$$E_h = E_m + 2.303\frac{\mathrm{RT}}{n\mathrm{F}}\log\frac{[\mathrm{Ox}]}{[\mathrm{Red}]}$$

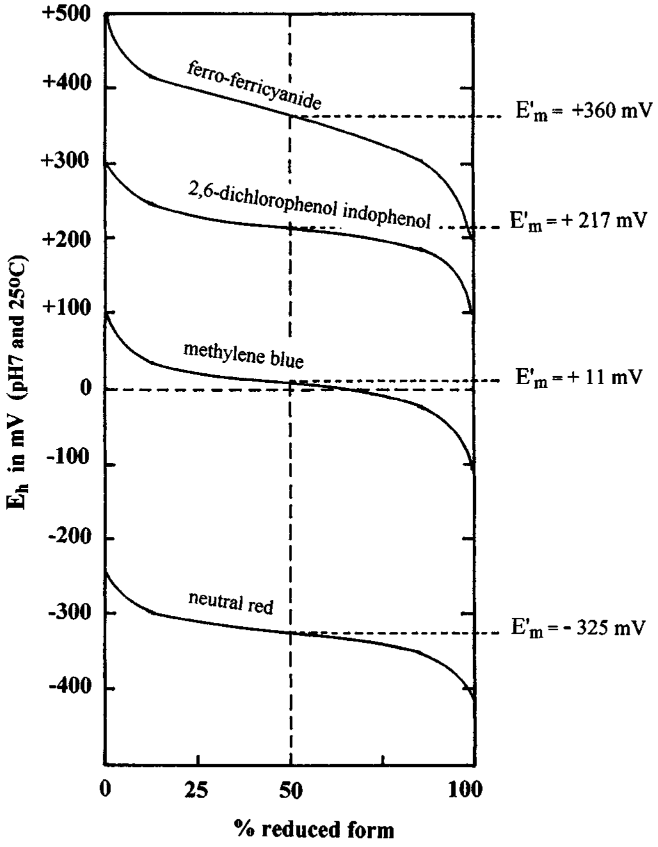

Figure 11-1 Relationship between the Redox Potential of a Redox Couple and Its Percentage Reduction. *Source:* Adapted with permission from J.G. Morris, *A Biologist's Physical Chemistry*, 2nd ed., pp. 322–359, © 1974, Edward Arnold.

At 30°C, the factor $2.303\mathrm{RT}/n\mathrm{F}$ is approximately equal to $0.06/n$ so that for a redox couple whose reductant loses two electrons when it is oxidized to the oxidant form, if when 50% oxidized its redox potential (E_m) was +100 mV, then when it was 99% in its oxidized form the potential (E_h) would be +160 mV, and when it was 99% in its reduced condition its E_h value would be +40 mV. Thus, for all intents and purposes, the working range of this $n = 2$ redox couple would be 120 mV.

11.1.3 Relationship between Redox Potential and pH

When protons are simultaneous participants in the operation of the redox couple, this can be represented in the form of the general equation given previously, ie,

$$a(\mathrm{Ox}) + b(\mathrm{H}^+) + ne^- \rightleftharpoons c(\mathrm{Red})$$

In these circumstances

$$E_h = E° + 2.303\frac{\mathrm{RT}}{n\mathrm{F}}\log\frac{[\mathrm{Ox}]^a[\mathrm{H}^+]^b}{[\mathrm{Red}]^c}$$

or,

$$E_h = E° + \frac{2.303\ \mathrm{RT}}{n\mathrm{F}}\log\frac{[\mathrm{Ox}]^a}{[\mathrm{Red}]^c} - 2.303\frac{b\mathrm{RT}}{n\mathrm{F}}.\mathrm{pH}$$

If unknown, the nature of the pH dependency of the couple can be deduced from plotting measured values of E_h against pH over the pH range of interest, when the slope of the line will equal $-2.303\ b\ (\mathrm{RT}/n\mathrm{F})$. If $b/n = 1$, then the slope $\Delta E_h/\Delta\mathrm{pH}$ will approximately equal 60 mV at 30°C. That is, the E_h will change by approximately 60 mV for every unit of pH change. Some E_h/pH relationships are complicated by the fact that the slope of the plot may change over various portions of the pH range. Indeed, there might be one part of the pH range in which E_h is independent of pH (when the plotted line will have zero slope) indicating that in this pH range there is no participation of protons in the redox couple (Figure 11-2). As noted previously, it is therefore essential to specify not only the measured E_h value, but also the pH at which it was determined (as well as the temperature).

In an attempt to avoid having always to record both E_h and the prevailing pH, Clark and his colleagues in the 1920s introduced the concept of *rH*. The reasoning was that if the pressure of hydrogen gas that would be in equilibrium with the redox couple were recorded, this would automatically accommodate any change in pH as well as E_h (and *rH* was suggested as most suitable for this purpose, being defined as the negative logarithm of the equilib-

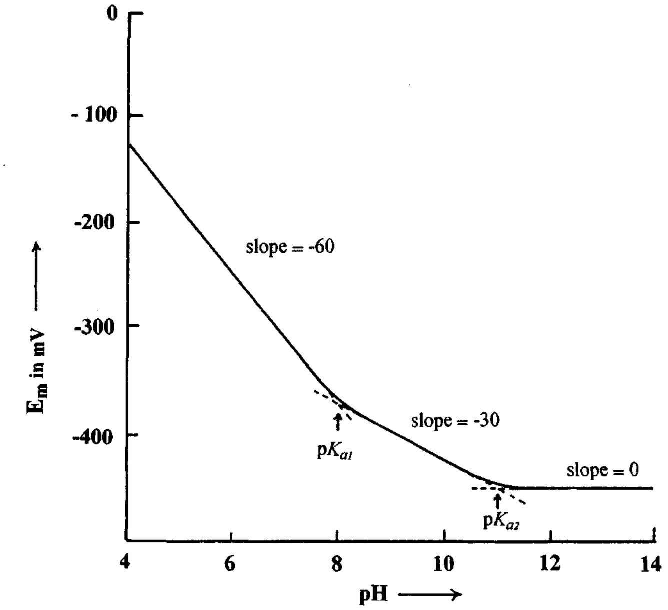

Figure 11-2 How pH Change Can Affect the Redox Potential of a Redox Couple. This is illustrated by a theoretical biprotic, weak acid (pK_a values of 8 and 11, and E_m at pH 7 of −300 mV), reduction of which, over the full pH range, is accomplished by the acceptance of two electrons per molecule. The slope of the relationship is most steep (−60 mV per pH unit increase) when two protons are simultaneously accepted. When only one proton is taken up per molecule along with the two electrons, then the curve is half as steep, and when no protons are implicated then the redox potential is independent of pH (in this instance >pH 12). *Source:* Adapted with permission from J.G. Morris, *A Biologist's Physical Chemistry,* 2nd ed., pp. 322–359, © 1974, Edward Arnold.

rium partial pressure of hydrogen). Unfortunately, because for many redox couples the relationship between E_h and pH was not as straightforward as for some others, *rH* values came to be inappropriately ascribed. Clark himself demanded[14] that the term *rH* should no longer be used because, "in brief, *rH* has become an unmitigated nuisance." However, should values of *rH* be encountered in some of the older literature, the corresponding values of E_h can be calculated provided the appropriate pH values are known,[34] for E_h at 30°C approximately equals $0.03(rH - 2\text{pH})$.

When reporting E_m (or $E°$) or E_h values that have been measured at pH 7 it is conventional to indicate this fact by assigning a superscript prime to the designation, eg, E'_m or $E°'$. Any other pH value (other than the standard pH = 0) is reported *verbatim,* eg, E_h at pH 6.4 = 234 mV. This enables comparative values of midpoint potentials of couples of biological interest (including redox dyes) to be listed as the more appropriate E'_m values (ie, relevant to systems at pH 7). Useful lists of these values have been published in several reviews.[14,28,34]

11.1.4 Redox Dyes

There exist a large number of organic chemicals liable to oxidation/reduction that are intensely colored in the oxidized or reduced state and either colorless or differently colored in the conjugate (opposite) state. For example, methylene blue (E'_m = +11 mV) is blue in its oxidized state but colorless in its reduced condition. Thus, if it were in solution to equilibrate with a redox system whose E'_h value was +50 mV it would confer upon it an intense blue color, for at this redox potential it would be 95% oxidized. However, in a system of E'_h −50 mV, methylene blue would be 99% in its reduced state and hence would impart no color to the system. Because redox dyes have been developed whose E'_m values cover the whole redox range of interest to biologists, the practice of using such substances as quick visual indicators of the prevailing redox potential of a solution is as usual as the similar use of pH indicator compounds.

The useful *transition range* of any redox indicator is that E_h span over which the fraction of the indicator that is present in its oxidized form changes from 90% to 10% of the whole (see Figure 11-1). However, here one encounters the first of several hazards in the indiscriminate use of such redox indicators because several of these compounds act simultaneously as pH indicators, whose acidic and basic forms also display distinctive colors.

To be effective, a redox indicator must rapidly and perfectly equilibrate with the redox potential of the test system; this also is not always achievable. Furthermore, the perfect redox indicator should be indifferent to the system whose redox potential it reports. Were it to be toxic to a biological component, or catalytic so that some reaction would occur in its presence that would not proceed at a significant rate in its absence, then it would be unacceptable. It should be noted too that redox indicators of the type described above undergo *reversible* redox transitions. This reversibility distinguishes them from other dyes whose reduction is almost irreversible, as in the case of the tetrazolium salts that are reduced to poorly soluble, intensely red or blue-colored formazans. Yet the near-irreversibility of the reduction of tetrazolium salts can also be advantageous. Thus Quastel and

Whetham,[66] when pioneering the use of methylene blue decolorization as a test of the ability of washed suspensions of bacteria to dehydrogenate various substrates, had to set up the reaction in evacuated Thunberg tubes to prevent aerobic reoxidation of the colorless leucomethylene blue. However, production of formazan from tetrazolium violet may be employed under aerobic conditions as a rapid test of the ability of a bacterium to utilize a range of substrates.[9] A rather more complicated situation exists in the case of resazurin which, over the E'_h range of +180 mV to +90 mV, is irreversibly reduced to the differently colored product resorufin. This, in turn, can serve as a reversible redox dye, being reducible ($E'_m = -51$ mV) to colorless dihydroresorufin. Thus, while resazurin (via its reduction to resorufin) has been employed under aerobic conditions as a relatively sensitive indicator of possible contamination of milk, it has also been widely used (via its further reduction with loss of all color) as an indicator of deoxygenation of anaerobic media.

11.1.5 Redox Buffering: Poising Effect

The redox potential of a redox couple (at a particular pH and temperature) is an *intensity term* whose magnitude (and sign) are only dependent on the nature of the couple, ie, its E_m value, and the ratio in which its oxidized and reduced forms are currently present. Put another way, how much of a redox couple is present will in no way affect its redox potential. Thus, a 70% oxidized redox couple will have the same E_h at a given temperature and pH whether its total concentration is 1 mM or 1 M.

On the other hand, the "resistance to change in redox potential" that is the basis of the redox buffering of a redox couple (also known as its poise or poising effect) will be determined by its total concentration as well as by the ratio in which its oxidized and reduced forms are present. The poising effect is thus a *capacity term* and the poising effect of a redox couple of a particular E_h will therefore be one thousand times greater if it is present at 1 M concentration than at 1 mM. The magnitude of its poising effect is manifested when the redox couple is titrated against a more potent oxidizing or reducing agent. Such a redox titration also makes it abundantly obvious that the poising effect of the redox couple is greatest when its oxidized and reduced components are present in equal amounts, ie, at its E_m value.

The relative redox buffering capacities, or poising effects, of all of the redox couples that are present in a system should (ideally) be taken into account when any attempt is made to interpret what is measured as the resultant redox potential of that system. Hewitt[28] illustrated this point by reference to the outcome of the addition of a redox dye to a biological system. At the prevailing pH and temperature, solutions of the dye would display the same E_h value irrespective of whether the dye was present in 0.001%, 1%, or 10% concentration. However, if a solution containing 100 g equiv. L^{-1} of the dye was mixed with a more reducing biological system containing only 1 g equiv. L^{-1} of the redox system responsible for its potential, then in Hewitt's words, "the dye could oxidize the whole of the biological system and still remain 99% oxidized, thus proving useless as an indicator of the condition of the system."

11.2 IN PRACTICE: REDOX POTENTIALS OF BIOLOGICAL SYSTEMS

11.2.1 Nonideal Behavior

The relationships described above would be displayed by ideal, reversible redox couples that interact with each other with no significant lag and that report their redox potentials via an inert electrode, again perfectly and immediately. The predictions that have been made also assume complete homogeneity of the aqueous systems involved, whose constituents are present in low concentrations that approximate to their activities.

Unfortunately, in practice many of these prerequisites are not met. Some redox couples are not easily reversible, some report potentials only sluggishly to measuring electrodes, and some interact only slowly with other couples except in the presence of specific oxidation-reduction catalysts. Also, in many foodstuffs such difficulties are compounded by their gross material and spatial heterogeneity, with phase differences and semisolid suspensions providing the conditions for the establishment of substantial gradients of redox potential over very small distances. When biological systems are involved, for example, in the case of microbial growth in a culture medium of any kind, it may appear that because very marked and rapid changes in redox potential can easily be measured, the redox buffering capacity of the system must be remarkably low. But it may be, as Hewitt[28] so graphically portrayed it, that "biological systems (can) behave, not as systems of fixed (redox) capacity or poising effect, but rather like a fountain-pen from which a small quantity of ink may be withdrawn for long periods at a slow rate. So that although the effective capacity of the biological system at any time may be very low, an almost inexhaustible process of reduction may proceed."

Attention to detail and technique can frequently help to overcome some of the difficulties encountered in obtaining reproducible and credible potential readings from a metallic measuring electrode. Jacob[34] emphasized the importance of careful preparation and cleaning of the electrode. When sluggish redox couples are involved, there are occasions when the addition to the system of a low (and hence not potential-distorting) concentration of another redox couple can cause this to serve as a mediator that rapidly reports to the electrode the potential generated by the test couple. Sometimes it can even be simpler than this. Thus, Rödel and Lücke[70] reported that to obtain stable and reproducible values when measuring E_h in sausage mixtures, it proved necessary to polish the surface of the platinum electrode prior to each measurement. Also according to Rödel and Lücke the hole punched for the insertion of the electrode "had to be wetted by a drop of 10 mM KCl to ensure good electrical contact between the product and the electrode. Without these precautions, 'phantom potentials' were frequently registered, or the time to reach stable reading was extended considerably."

By such means one can employ a platinum electrode, or its equivalent, to register an electrical potential at any site in an aqueous medium no matter how complex and heterogeneous this is. The difficulties arise in interpreting the causation and significance of the potential so measured. It is a temptation to attempt rigorously to apply the same reasoning that informs our understanding of the ideal systems outlined above. Unfortunately, this, more frequently than not, is a temptation that should be resisted. The chief danger is to be so beguiled by the reproducibility of measurement of a E_h value as to believe that this value must have some independent significance, that it serves as a determinant rather than as a reporter of the oxidized/reduced status of the system. If prone to this delusion, the best cure is to ask oneself what it is that is actually being measured. Will all of the redox couples potentially present in the complex substrate be so perfectly interactive that your instantaneous measurement will be of the redox status of the dominant (most oxidative couple) present in such concentration as to overwhelm all subsidiary couples? Or is it conceivable that the measured E_h value may take no account of the existence of some of the redox couples that are possibly present, because they are intrinsically sluggish (nonelectromotively active at the measuring electrode), or nonreversible, or for some other reason not accessed by the measuring device. The history of the measurement of redox potentials in bacterial cultures well illustrates these concerns.

11.2.2 Redox Potentials in Bacterial Cultures

Potter[65] was possibly the first to draw attention to the fact that a growing microbial culture generated a negative (ie, reducing) potential with respect to the uninoculated medium of initially identical composition. Thereafter, many investigators demonstrated the progressive fall in redox potential associated with growth and reproduction of bacteria in a variety of defined and nutritionally complex media.[2,11,13,23,28,32,34,35,44,59,64,68] This was noticeable even during the growth of aerobic bacteria in well-aerated media, though on the cessation of growth, the measured E_h of these batch cultures would generally rise again. This pattern of decrease in potential from an initial E_h value of somewhere around +350 mV to perhaps –50 mV with a subsequent rise during the stationary phase of batch culture contrasts with the behavior of cultures of facultative anaerobes.[28] In batch cultures of the latter, whereas, although initially the rate of decrease in E_h may be similar to that displayed by cultures of aerobic bacteria, after passing through a zone (around +270 to +200 mV) where the rate of decrease of E_h is slower and possibly indicative of some enhanced poising effect, thereafter the pace of E_h fall quickens again and the potential can attain a much lower final value (eg, –350 mV). Obligately anaerobic bacteria may not be able to initiate growth in oxygenated media (indeed, it is this that mostly distinguishes them from facultative anaerobes), but they may do so when the oxygen is removed and some reducing agent(s) is incorporated in the medium to decrease its initial E_h. Thus, addition of dithiothreitol can cause the E'_h of a medium to fall to quite low values (–250 to –300 mV). Once growth commences, however, then the E_h may rapidly diminish, in some instances (for example, when hydrogen is metabolically generated) achieving values more negative than –400 mV.

Such observations are very easily made and their reproducibility persuades one that they can be helpfully informative. In several instances a change in metabolic behavior in the course of bacterial culture growth is reliably signaled by a simultaneous change in the measured E_h. Monitoring E_h changes in microbial cultures, or alternatively controlling the E_h of a microbial culture in order to stabilize or amplify some desirable growth characteristic, has by now achieved respectability.[32,38,46,80]

Emde and Schink[18] achieved an increase in propionate formation by *Propionibacterium freudenreichii* by growing it in a 3-electrode poised-potential amperometric culture system, which employed either anthraquinone 2,6-disulfonate (poised E_h −224 mV) or cobalt sepulchrate (poised E_h −390 mV) as mediator. Yet in very few natural instances does the experimenter have any knowledge of precisely what is being measured by his recorded E_h value. Gillespie,[23] who in 1920 first applied the term *redox potential* to the electrode potentials measured in bacterial cultures, took comfort in the observation that "(their) biological significance may be capable of demonstration even if (their) chemical significance may not."

Hewitt[28] said that "definite electrode potentials are developed in cultures, but we are not fully aware what substances are responsible for these potentials in many cases, nor what reversible oxidation reduction systems are actually being measured." In her classic textbook on bacterial metabolism, Marjory Stephenson[78] picked her words very carefully when she wrote, "The fact that certain growth phenomena, eg, the germination of the spores of *Cl. tetanum* can occur only in conditions which give certain potential readings with the electrode, shows that the presence of substances associated with (not necessarily causing) these readings conditions the growth phenomena in question." There can be no doubt that what is being measured as the instantaneous platinum electrode potential in a bacterial culture is the resultant of the microorganism's metabolism of substrates provided by the growth medium and atmosphere, the outcome being a trend toward enhancement of the reducing intensity of the growth environment (sometimes amplified by its simultaneous acidification). An instantaneous E_h reading under these circumstances is at best a snapshot of a dynamic system; the crucial question is whether the electrode is actually recording that which is sensed by the microorganism. This becomes an especially important question when the primary determinant of the measured redox potential appears to be oxygen (aeration).

11.2.3 Oxygen and Redox Potentials in Bacterial Cultures

Being the most avid, pervasive, and generally available of biologically active oxidants, considerable attention has been paid to the fate of oxygen in bacterial cultures and to the influence that it has on the E_h values measurable in these cultures.[27,46,58,85] Were it to be reduced according to the following equation:

$$O_2 + 2H^+ + 4e^- \rightarrow 2OH^-$$

the E'_m value of the oxygen couple would be +810 mV at pH 7, and in an aqueous buffer at this pH, which was saturated with air the E_h due to oxygen would be expected to be +806 mV.[63] In practice, when using a suitably prepared platinum electrode, the E_h of a 0.666 M phosphate buffer pH 7 saturated with air at 1 atm was found to be +630 mV, while the E_h of a meat broth–peptone medium (pH 7) with a similar dissolved pO_2 of 0.2 atm, was only about +400 mV.[34] The above equation is therefore not an adequate representation of the fate of oxygen as oxidant in aqueous systems. Furthermore, besides its oxidative effect on the redox components of the medium, the possibility exists of direct interaction of oxygen with the measuring platinum electrode.[36] Experimental measurement of the relationship between the partial pressure of oxygen dissolved in nutrient media and the registered E_h value suggested that the E_h was proportional to the logarithm of the pO_2, the slope of the straight line relationship being 60 mV/log pO_2. On this basis, Jacob[34] calculated that the pO_2 in a culture of growing aerobic bacteria would be about 10^{-8} atm and in a growing culture of facultatively anaerobic bacteria about 10^{-12} atm. However, it is evident that the actual relation between pO_2 and E_h will depend on the chemical composition of the culture medium, which will change progressively due to the metabolic activities of the microorganisms whose growth it supports. Ishizaki et al.[32] reported that in bacterial fermentations a 10-fold change in pO_2 resulted in E_h changes of between 100 and 115 mV. Lund et al.[51] found that in an uninoculated peptone, yeast extract, meat extract medium containing thionine at 2 mg L^{-1}, there was a linear relation between pO_2 and E_h, such that, at pH 6.8 to 7.0, the medium responded to a 10-fold increase in pO_2 by a 138 mV rise in E_h.

Such extrapolations implicitly assume that in any bacterial culture, oxygen, when present, will be the dominant oxidant, it being ideally and instantaneously capable of accepting electrons available for donation by all couples of lesser E_h. This would be consonant with the view[85] that a major redox couple can swamp all other minor redox couples and therefore dictate the measured value of the culture redox potential. Were this the case, during an aerobic fermentation, the dissolved oxygen concentration should indeed be accurately mirrored in the measured value of the culture redox potential. Such an assumption would appear to be authenticated by the fact that in many microbial cultures it is possible to control the E_h value by adjusting the rate of aeration, or conversely, by controlling the E_h to regulate the rate at which air need be supplied. An interesting example of the practical utility of E_h control serving as proxy for dissolved oxygen control is provided by the way in which by maintaining at +40 mV the E_h of the surface aerated zone of an otherwise anaerobic reactor treating piggery waste water, aeration could be made intermittent and less costly while satisfying the objective of containment of foul odors.[4] Yet, it is not always the case that a simple equation can be made between dissolved oxygen and measured E_h for there are many examples of bacterial cultures being sustained at E_h values other than those that would be representative of the dissolved oxygen concentration in the medium. Kwong and Rao[45] showed that by fortifying the culture medium of *Corynebacterium glutamicum* with reducing agents such as dithiothreitol, it was possible to decrease the culture redox potential under aerobic conditions without affecting the high dissolved oxygen concentration. Thompson and Gerson[80] used a 50 V DC power supply and neutral red addition (at 0.25 μg ml^{-1}) to control electrochemically the redox potential of an aerated culture of *Escherichia coli* B/5 at +380 mV when, uncontrolled, the measured E_h would have been considerably higher. These, and other examples, counsel against (1) adopting a simplistic view of the relationship between dissolved oxygen concentration and measurable E_h in microbial cultures and other biological systems, and (2) assuming that all of the redox couples present in a biological system are perfectly reversible, highly electromotive, and capable of instantaneous interaction and equilibration.

11.2.4 Is It the Measurable Environmental E_h That Is Sensed by a Bacterium?

The point has been made that E_h has no existence independent of the couple whose redox intensity it reports. Also, in an ideal system of instantaneous equilibration of all available redox couples, it is that couple of greatest poise that will dictate the measured E_h of the system. Yet, even if this could be relied on, and it has been seen that such ideal behavior of mixtures of redox couples in biological systems cannot be assumed, the important question is whether it is this prevailing environmental E_h that is directly sensed and responded to by a bacterium. Some insight into the complexity of the problem can be gained by a consideration of the reaction of a bacterium such as *Escherichia coli* to oxidative stress caused by exposure to oxygen, and also of the regulatory mechanisms employed by this organism so to control gene expression as to exploit respiratory oxidants to best advantage.[33]

In its oxidative stress response, *Escherichia coli* responds differentially to hydrogen peroxide (via the OxyR regulon) and to superoxide anion (via the SoxRS regulon) rather than just to the high E_h that would simultaneously be sustained in its environment. Again, certain other bacteria respond to the oxidative stress provoked by aerobic exposure to benzylviologen and similar chemicals by accumulating 2-methylbutan-1,2,3,4-tetraol 2,4-cyclopyrophosphate, which in some undisclosed manner may serve as an antistressor,[60] although what these organisms perceive as the inducing stress is not known.

In the shift from aerobic to anaerobic respiratory and fermentative metabolism at least three gene regulatory systems are implicated in *E. coli*, viz. Fnr, NarL/Nar X, and ArcB/ArcA. The E'_m of nitrate/nitrite at pH 7 is +420 mV and, in the absence of oxygen, were nitrate to be available to *E. coli*, it would be the first of the alternative respiratory oxidants to be consumed. This primacy of nitrate usage is ensured by the *nar*L gene product acting in concert with nitrate and molybdate as a positive effector of transcription of the operons encoding the necessary nitrate reductase and formate dehydrogenase and repressing the expression of other anaerobic terminal reductases whose substrates have lower E'_m values, eg, fumarate/succinate E'_m +31 mV. Yet there is simultaneous overarching control from the FNR regulatory protein, which is functional only when oxygen is virtually absent from the environment. The presumption is that FNR (which is an Fe-S protein) is activated directly or indirectly by reduction but it is not known what is the electron donor in that reaction.[26]

The picture that emerges from these and other findings is that environment E_h can very possibly be sensed by bacteria via the altered redox status of some key component of their electron transport chains or (transcriptional) regulatory systems. For example, by displaying "redox taxis," *E. coli* (normal cytoplasmic midpoint redox potential < −250 mV[82]) demonstrates that it is able to sense the redox state of its surroundings and so swim to a favored position in a spatial redox gradient.[79] For this purpose a flavoprotein (Aer) serves as a redox sensor together with a serine chemoreceptor protein (Tsr), the pair also playing the same roles in the aerotaxis also displayed by the bacterium.[67] Other bacterial redox-regulatory switches that are responsive to oxidative stress are activated by a variety of mechanisms so that in some instances a specific oxidant can serve as the trigger of consequences that at first sight would appear to be more generally E_h determined. Thus OxyR senses peroxide-induced stress in *E. coli* via the oxidation of a cysteine thiol group to a sulfenic acid species, while SoxS that is responsive to superoxide radical-induced stress, uses a [2Fe-2S] cluster as the sensor. The FNR protein of *E. coli* employs cysteine-linked [4Fe-4S] cluster, while the FLP of *Lactobacillus casei* is a homodimeric protein wherein each subunit can on oxidation form an intramolecular disulfide bond.[24] Other redox responsive components of bacterial regulatory systems include the FixL hemoprotein of the two-component regulatory system for N_2-fixation in *Sinorhizobium meliloti,* and the NifL modulator of NifA-activated N_2-fixation in *Azotobacter vinelandii* that resembles Aer in using FAD as the redox sensor.[24] The situation is further complicated by the intimate relationship that exists between the redox status of the bacterial cell and its transmembrane proton motive force, which in turn reflects the "intracellular energy level" of the cell.[79]

There are instances when changes in environmental E_h affect bacterial metabolism even more indirectly. One example is provided by the particularly potent bacteriostatic action of nitrite on some food spoilage organisms. It remains a matter of some debate whether the additional enhancing effect of reagents, such as ascorbate or iron salts or thiols, is in this instance due to the formation of nitric oxide and iron-thio-nitrosyl complexes.[53,62] Pessimistically, one might reflect that any one organism could respond very differently to a particular E_h depending upon the chemical nature of the dominant poising couple.

11.2.5 Tentative Conclusions

Redox potentials can be easily and reliably measured in all manner of biological systems but, except in the most simple, it is rarely evident what (dominant) redox couple dictates this measured value. However, if one accepts the limitations of interpretation, then the measured E_h value and the rate and extent whereby it changes in response to events within the system can prove to be usefully informative. It is for this reason that E_h values measured in complex biological systems have variously been termed phenomenological values, empirical values, pragmatic values, even instrumentational values. *It cannot be stated too frequently that an* E_h *value reports on conditions, it does not of itself create them.*

11.3 REDOX POTENTIALS AND FOODSTUFFS

11.3.1 General Observations

In the context of food preservation and safety, the measurement of the redox potential of a foodstuff has the twin aims of (1) determining whether the substrate is capable of supporting growth of various classes of contaminant microbes, and (2) suggesting how the background redox potential might be so adjusted by the addition of a suitable oxidant or reductant as to make the substrate uncongenial to the likely microbial contaminants while not affecting its palatability and attractiveness as a foodstuff.[12] In the case of obligately aerobic microbes, the question might be asked whether the measured E_h value reflects the availability of dissolved oxygen so that it could signal when this achieves too low a concentration to sustain their growth. On the other hand, those obligate anaerobes that will not initiate rapid growth in an insufficiently reducing environment might be excluded if the E_h is poised at a sufficiently high value. In this context it should be noted that the redox buffering of certain foods is much higher than others due to their larger content of oxidants and reductants, so that microbial contamination that might cause a very marked change in the E_h of some less strongly redox buffered foodstuff might not significantly affect the E_h of another that is much more strongly poised. Such considerations will determine the nature and quantities of which preservative oxidants or reductants should be incorporated into the foodstuff and/or in what atmosphere this should be packaged in order to maximize its shelf life.

Anoxic packaging (under vacuum, or in carbon dioxide and/or nitrogen) will evidently exclude contaminant obligate aerophiles and will encourage the development of relatively low E_h values. To the same end, it has been suggested that copper(II) ascorbate could serve as an effective preservative in foods and beverages,[25] chiefly because of its ability rapidly to remove residual and headspace oxygen without generating damaging oxygen free radicals. A complication arises from the fact that some foods are of low E_h value even though they have been packaged in air. Thus, the measured redox potential of some delicatessen items was found to be as low as –198 mV to –23 mV, giving rise to concern that they might be more likely to pose a risk of botulism.[77] It has also been noted that the measured heat resistance of some facultatively anaerobic bacteria (including *E. coli* O157:H7, *Salmonella enteritidis,* and *Listeria monocytogenes*) is more than eight-fold greater when they were grown, heated, and (especially) recovered anaerobically rather than aerobically.[22] Thus, George et al.[22] concluded that "anaerobic enumeration should be used to give a realistic measure of the ability of the heat process to eliminate facultatively anaerobic pathogens from foods that are packed under vacuum or low oxygen modified atmospheres or that have a low redox potential despite being exposed to air."

In oral medicine, there is precedence for the antimicrobial use of various redox dyes. For example, topically applied methylene blue was employed in the treatment of periodontal disease. By raising the local E_h it inhibits the growth of causative, obligately anaerobic, bacteria.[21,84]

The pragmatic nature of any E_h measurement that is made in a heterogeneous system has already been emphasized. Unfortunately, this is not the only problem that is encountered by the food microbiologist who is also aware that many foods present a mosaic of possible habitat domains capable of supporting distinctive microbial communities. As time passes, these can change in population composition both due to progressive changes in the physicochemical characteristics of the substrate but also as their activity domains variously interact.[10] Shifting gradients of redox potential may also exist, most obviously paralleling gradient(s) of oxygen diffusion into the depth of a foodstuff. Despite these complications, it has generally been hoped that there will prove to be limiting E_h values or at least ranges of E_h, which, if not achieved, will ensure that growth of certain bacteria cannot occur, however suitable in other respects the substrate might be. Thus, in tests of strains of *Pseudomonas fluorescens* and *Salmonella* serotypes isolated from poultry, it was discovered that at pH 7, the limiting E_h for growth of the *Pseudomonas* was +80 mV.[42] In the same study, it was found that *Clostridium perfringens* affected growth of the pseudomonad in mixed culture at low values of E_h but not at an initial E_h of +200 mV, leading the authors to propose that, "with the increasing trend toward vacuum packaging of meats and poultry, the significance of oxidation-reduction potential on associative growth of aerobes and anaerobes warrants further study."

Plotz and Geloso[64] reported that any one of five clostridia rapidly lowered the E_h of anaerobic meat broth from an initial +80 mV to less than −200 mV at pH 7, although rapid growth occurred only when the E_h of the medium lay between −6 mV and −436 mV. Knaysi and Dutky[40] found that while growth of a butyric clostridium would not take place under aerobic conditions at +300 mV, it would do so when the dissolved oxygen tension was low and the medium was poised at +335 mV by the addition of ferricyanide. On the other hand, Kliger and Guggenheim[39] found that *Clostridium welchii* (ie, *C. perfringens*) would grow in a medium at 95% of the air-equilibrated oxygen tension provided that sufficient ascorbate was present to poise the medium at −125 mV. The larger the inoculum of vegetative cells that was used, the higher (up to about −90 mV) was the initial E_h that was compatible with growth of several pathogenic clostridia.[68] Fildes[20] had earlier pointed out the likelihood that the E_h in the vicinity of a dense, nondispersed inoculum of clostridial cells fell much more rapidly that when the same inoculum was more evenly distributed throughout the medium. Morris and O'Brien[58] reviewed these and other reports: (1) of the lowest values of E_h developed in growing clostridial cultures, (2) of the upper limits of E_h at which such growth could be initiated, and (3) of the particular situation in which dissolved oxygen was the determinant of the measured E_h. They concluded that in the case of any *Clostridium*, the magnitude of the limiting culture E_h above which the vegetative organism will not grow (variously reported as being between +30 mV and +250 mV), will not be invariant but will be determined by several factors including the composition of the medium, the inoculum size, whether or not the E_h is artificially maintained and, if so, by what agency. Quite early on, it was recognized that media components that were not themselves redox active, and a variety of other stress-inducing factors (including nutrient deprivation, suboptimal pH values) could affect the outcome. Thus, Mead[54] found that with *Clostridium perfringens* growing in a complex medium in the presence of 0.5% NaCl, the upper limit of E_h compatible with growth was +194 mV, but as the concentration of NaCl was further increased the limiting E_h decreased until at 5% NaCl its value was +66 mV.

In the case of clostridia, and especially *Clostridium botulinum* (see below), interest has centered not only on the range of E_h values over which the growth of vegetative cells may be initiated, but also on the oxidation-reduction potential limits that dictate whether or not their endospores can germinate. In general, the germination of spores of *Clostridium* species is less oxygen sensitive than is the growth of the emergent vegetative bacteria. However, in anoxic conditions a marked fall in culture E_h can accompany the germination of a large spore inoculum, thereby providing the conditions suitable for the multiplication of the outgrowing vegetative cells. Thus, following inoculation of a culture medium with 3.4×10^7 *C. butyricum* spores ml^{-1}, the culture E_h fell from +200 mV to less than –200 mV within 1 hour,[17] a fall that was seemingly associated with the fermentative production of hydrogen gas. Inoculum size may also influence the value of the determined growth-limiting E_h because the larger the number of spores/organisms employed, the greater the likelihood that among them there will be variant individual(s) capable of better tolerating adverse redox conditions.[50]

Although the majority of such studies have been performed with laboratory culture media, there are circumstances when the findings are mirrored in observations made with foods.

11.3.2 Milk and Dairy Products

The dairy industry has notably employed various redox dyes in rapid qualitative tests of milk purity. Thus, because raw milk has an E'_h of about +200 mV under aerobic conditions, added methylene blue (E'_m = +11 mV) will not be reduced and hence decolorized unless the milk is significantly contaminated with unwelcome bacteria. Resazurin in its reduction to resorufin (see Section 11.1.4) is a rather more sensitive indicator of the presence in milk of mastitic pus and tissue cells. Though hopes have been expressed that the rates of reduction of some of these redox indicators might prove to be proportional to the load of microbial contaminants in the dairy product, this would not appear to be the case. Furthermore, the rates of reduction of various redox dyes by foodborne bacteria vary greatly between different bacterium/dye combinations, so that with any one dye, there is considerable variation with each organism and between different organisms.[47] Care must therefore be taken in interpreting the findings made in such dye reduction assessments of microbial contamination.[43]

Somewhat surprisingly, sterilized milk is a poorly poised medium. The redox potential of milk sterilized by heating at 115.5°C for 15 minutes in evacuated bottles was –290 mV, but milk similarly heated in open bottles in air had an E_h of –30 to +10 mV. Milk heated in such a way as to conserve/produce reducing components that are evidently sensitive to aerobic oxidation did not support as rapid growth of *Bacillus cereus* 201 and *B. brevis* 58 from small spore inocula as did milk heated in open bottles.[29] The method used for sterilization and the oxygen permeability of the final packaging may both affect the redox potential of the product. For example, samples of UHT single cream in Tetrapak© containers prepared aerobically by two different processes displayed mean E'_h values of +286 mV and +222 mV, respectively (Dr. B.M. Lund, personal communication). To achieve this degree of accuracy and reproducibility of redox potential determination in an aerobic system, it was necessary before each measurement to clean the measuring electrode and restandardize it in aerated phosphate buffer.[51] Even so, the prepared and oxygen-responsive electrode then had to be immersed in the cream under a stream of oxygen-free nitrogen for between three and five hours before stable readings were registered.

In making measurements of redox potential changes in ripening cheddar cheese Davies et al.[16] found that oxidative taints and oiliness did not appear when the E_h at pH 6.5 was less than +300 mV. On the other hand, anaerobic faults such as *rusty spot* did not appear when the E_h at pH 5 was above –100 mV. Gassy fermentations were preventable by adding oxidants. In this respect, nitrate was particularly effective, though this was more likely due to nitrite and its products being particularly inhibitory to the growth of organisms such as *Clostridium tyrobutyricum*. The electrode potential measured in the interior of a ripening cheddar cheese followed a predictable course indicative of the initial establishment of an almost pure culture of *Streptococcus lactis* followed in later stages by a lactobacillus.[28] The development and variations in gradient(s) of E_h within cheeses could also be informative. In Brie cheese, oxygen gradients were steep in the uppermost 2 mm of the cheese and below this level the cheese was essentially anaerobic. A pH gradient deriving from lactic acid production extended from acid (pH 5.5) in the center to more neutral pH values toward the surface. Each of these contrary gradients was established by microbial activity, and just beneath the fungal rind, the pH and oxygen tension were mutually appropriate for the growth of *Listeria monocytogenes*.[10]

11.3.3 Meats and Meat Products

Barnes and Ingram[5] reported that the redox potential of horse muscle fell after death from +250 mV to –130 mV. At the onset of rigor it was 0 mV to –50 mV, and below –36 mV it supported rapid multiplication of *Clostridium perfringens*. This fall in E_h of carcass meat is due to the continued activity of indigenous enzymes during the cooling period. Simultaneously, the oxygen reserves in the depth of the muscle become depleted, and the pH decreases. Thus, for a period, the E_h remains relatively high as the pH becomes more acidic, allowing time for the carcass to cool to a safe temperature before conditions become appropriate for the growth of obligately anaerobic bacteria. Thereafter, much depends on the manner in which the meat is processed and packaged. Methods for the measurement of E_h in meat products have been published[48,71] and there is ongoing interest in the preservative role of nitrite[31] (see also Chapter 9).

Madden and Bolton[52] measured E_h as well as pH and numbers of aerobic, microaerophilic, and anaerobic microorganisms in fresh beef and a chicken liver paté, which had been vacuum packed prior to refrigerated storage. The beef showed a rapid fall in surface E_h, which then stabilized until the anaerobic microflora (largely lactic acid bacteria) was established. Analyses undertaken on the exudate from commercially produced vacuum packs of beef primal cuts showed significant correlations between the numbers of both anaerobic and microaerophilic organisms and negative E_h values. They concluded that E_h is a significant parameter affecting, and affected by, the growth of anaerobes, and that its measurement may serve as an indicator of their number. In minced fresh meats that are loosely packed under an air-permeable film, the surface E_h will remain relatively high and the major bacterial contaminants are likely to be pseudomonads and *Brochothrix thermosphacta* plus lactic acid bacteria. Antioxidants that rely on bisulfite and sulfite for activity delay microbial spoilage of certain meat products, and other antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene used to counter rancidity, are now known to inhibit the growth of Gram-positive bacteria.[72] Wirth and Leistner[86] suggested that an initial E_h between –20 mV and –150 mV is optimal for canned meats, with additives such as nitrite and ascorbate contributing to the poising effect. The shelf life of pasteurized meat products that are packaged in hermetically sealed containers is likely to be compromised by the growth from viable spores of *Bacillus* as well as *Clostridium* species, with *B. subtilis, B. pumilus,* and *B. licheniformis* predominating.[7] However, from studies undertaken with Bologna-type sausage mixtures infected with these *Bacillus* species, Rödel and Lücke[70] concluded that the microbiological stability of pasteurized meat products cannot be predicted from measured redox potentials alone.

11.3.4 Redox Potential, Oxygen Tension, and the Probability of Growth of *Clostridium botulinum*

For obvious reasons, much attention has been focused on determining those properties of foods (fish, meats, milk, canned products) that predispose them to be unsuitable media for spore germination, and subsequent vegetative cell multiplication, of *C. botulinum* types A and E. One question has been whether a reliable ceiling value can be placed on the oxidation-reduction potential span that is compatible with growth of this pathogen from spore inocula in a given medium.[1,50,56,75,76] The evident inability of *C. botulinum* to grow in the presence of even modestly high concentrations of oxygen similarly caused assessments to be made of the limiting oxygen concentration for vegetative cell growth. Early estimates placed this limit at about 4% to 9% of normal atmospheric oxygen tension,[15,55] although germination of spores of *C. botulinum* type E can, under certain circumstances, occur in aerated media.[1]

Milk sterilized by autoclaving at 121°C for 18 minutes registered an E_h value of +234 mV which, due to autoreduction, decreased to +189 mV in six days of standing. When the newly sterilized milk was inoculated with 2×10^3 to 5×10^3 spores ml^{-1} of *C. botulinum* type 62A, there was a lag time of seven days before growth began.[37] More prolonged (30 minutes) heating of the milk at 121°C yielded a product with an initial E_h of +192 mV, which fell during six days standing to +129 mV. In this medium, the lag time for growth from the same-sized inoculum of *C. botulinum* type 62A was only five days. In both cases, inoculation of the milk samples three or five days after sterilization led to a shorter lag phase than when the medium was inoculated two hours after autoclaving. This led Kaufmann and Marshall[37] to conclude that the autoreductive property of sterilized milk played a major role in development of conditions suitable for growth therein of *C. botulinum* type 62A.

These and other early studies suggested that whether or not a particular culture medium was capable of supporting growth of *C. botulinum* (all other factors being propitious) might be as much dependent on the nature of its major oxidant as on its measurable E_h value. Thus, when a *C. botulinum* type E was injected (at 100 spores g^{-1}) into the muscle of freshly smoked herring, which was vacuum packed and then stored at 15°C, botulinum toxin was formed within three days despite the fact that the E_h of the muscle remained almost constant in the range +200 to +250 mV.[30] This despite the fact that in normal culture media the highest possible E_h at which growth of *C. botulinum* type E was initiated was considered to be +200 mV at pH 7. Indeed, Ando and Iida[1] had previously reported that although large inocula (10^8 spores ml^{-1}) in an aerated culture medium of E_h +293 mV germinated, they showed no further post-germinative development. However, in the herring muscle the major oxidant would be not oxygen but trimethylamine oxide which can actually be utilized by *C. botulinum* type E as an electron acceptor.[49]

The presence of a preservative, for example, NaCl,[19,50] can significantly affect the E_h range within which growth of *Clostridium botulinum* may be initiated. Segner et al.[73] found that with an inoculum of 2×10^5 viable spores ml^{-1}, 5% w/v NaCl inhibited the growth of three strains of *C. botulinum* type E for one year during incubation at 16°, 21°, or 30 °C. When trypticase soy broth containing 4% NaCl was inoculated with 3×10^6 spores ml^{-1} of *C. botulinum* type E, if the medium was adjusted to E'_h −190 mV, growth was apparent after 48 hours at 30°C, but if the E'_h was −80 mV, no growth occurred during 200 hours incubation at this temperature.[74] Lund and Wyatt[50] found that the probability of growth in five days from a small spore inoculum of *C. botulinum* type E strain Beluga introduced into a culture medium of pH 6.8–7.0 containing 0.1% NaCl, was not significantly different at E_h +60 mV (with introduced air) than at −400 mV (anaerobic). Increasing the E_h above +60 mV, however, decreased the probability of growth, so that between +122 mV and +164 mV, this was 10^5-fold less than that evidenced at −400 mV. Raising the NaCl concentration to 3.25% and 4% in the anaerobic medium of E_h −400 mV decreased the probability of initiation of growth by factors of 10^2 and 10^4, respectively.

Riemann[69] suggested that the success of any preservation method with respect to infection of food by *C. botulinum* could be gauged from its effect on the probability that one botulinum spore would grow out and so produce toxin. Lund[49] and colleagues adopted this principle in computing the probability (*P*) for initiation of growth from a single spore of *C. botulinum* when small spore inocula were employed to infect various culture media. Recognizing that it is not possible to make simple correlations between dissolved oxygen concentrations and E_h values in dif-

Table 11-1 The Effect of Partial Pressure of Oxygen on the Redox Potential of the Medium and on the Probability (*P*) for Initiation of Growth from a Single Spore of *Clostridium botulinum* type E

pO_2 (atm × 10^{-2})	E_h (mV)	*Log* P *for Growth in Days*			
		2	*3*	*5*	*14*
1.67	+294[a] +328[b] (±14)	<−5.41	<−5.41	<−5.41	<−5.41
1.30	+280[a]	<−5.41	−5.36 (−4.52 to −6.80)[c]	−5.01 (−4.30 to −5.95)	−4.69 (−4.08 to −5.39)
1.12	+271[a]	<−5.41	−5.36 (−4.52 to −6.80)	−5.36 (−4.52 to −6.80)	−5.05 (−4.39 to −5.99)
0.79	+250[a]	<−5.41	−4.77 (−4.14 to −5.56)	−4.10 (−3.56 to −4.63)	−3.87 (−3.33 to −4.40)
0.72	+245[a]	−2.63 (−2.03 to −3.30)	−2.22 (−1.65 to −2.84)	−1.14 (−0.49 to −1.80)	−0.97 (−0.32 to −1.62)
0.39	+208[a]	−4.33 (−3.73 to −4.95)	−0.63 (−0.04 to −1.30)	−0.63 (−0.04 to −1.30)	−0.63 (−0.04 to −1.30)
0.21	+170[a] +189 (±12)[b]	−0.55 (+0.06, −1.24)	−0.55 (+0.06, −1.24)	−0.55 (+0.06, −1.24)	−0.55 (+0.06, −1.24)
0	Approx. −400[d]	0 (+0.48, −0.48)	0 (+0.48, −0.48)	0 (+0.48, −0.48)	0 (+0.48, −0.48)

Note: Measurements were made in peptone-yeast-sucrose-starch (PYSS) medium containing thionin, 0.002 g L^{-1}, pH 6.8–7.0.

[a] Estimated from a standard curve relating pO_2 to E_h.

[b] Determined by measurement on uninoculated vials of medium.

[c] Figures in parentheses are 95% confidence limits.

[d] Medium at E_h = −400 mV contained cysteine-HCl, 0.5 g L^{-1}, and titanium III citrate, 1.1 mM.

Source: Adapted with permission from B.M. Lund, Quantification of Factors Affecting the Probability of Development of Pathogenic Bacteria, in Particular *Clostridium Botulinum*, in Foods, *Journal of Industrial Microbiology*, Vol. 12, pp. 144–155, © 1993, Macmillan Press, Ltd.

ferent media, they further recommended that both parameters should routinely be measured and recorded. Table 11-1 reproduces their findings concerning the effect of partial pressure of oxygen on the redox potential of the culture medium and on the probability of initiation of growth from a single spore of *C. botulinum* type E. These and similar observations made by other workers explain why oxygen should be expelled, and good reducing conditions established, before an attempt is made to quantify the effects of any other possible preservative on the growth of *C. botulinum* (an E'_h of -200 mV to -300 mV, or lower, was recommended.[49]

11.4 CONCLUDING REMARKS

Redox potentials in foods are relatively easily measured but the values so obtained are difficult to interpret wisely. They are the result of contending oxidative and reductive tendencies attributable to components that are present in differing concentrations (and hence poising effect) and which may not be in perfect equilibrium nor equally electromotive at the measuring electrode. Yet the alternative use of redox dyes to indicate redox potentials is fraught with so many difficulties that, for quantitative purposes, use of electrode-based measuring instruments is obligatory. This imposes the further requirements (1) that the measuring electrode be so prepared and calibrated that it gives stable and reproducible readings, and (2) that a foodstuff is tested in a manner that does not cause any change in the potential that is to be measured (eg, a food should be tested in the atmosphere in which it is packaged with precautions being taken to avoid ingress of air when this has previously been excluded).

Despite these concerns, measured E_h values, and time-dependent changes therein, can prove helpful as one of several indicators of the susceptibility of a food to microbial infection and of the course of consequential microbial activities, providing information, which, in turn, can inform choice of an appropriate combination of preservative measures. At the same time, it would be unwise to use redox potential information in isolation to predict food safety, or to rely exclusively on control of redox potential as the means of preventing growth of specific microorganisms.

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12 Microorganisms and Their Products in the Preservation of Foods

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12.1 INTRODUCTION: HISTORICAL PERSPECTIVE

Use of biocontrol (biological preservation) in foods or the use of microorganisms and their products in the preservation of foods predates recorded history. This earliest aspect of food biotechnology evolved with the global development of fermented foods and beverages. Along with salted and dried foods, fermented foods represent the first processed foods. Through serendipity and realization passed down through generations, people lacking scientific understanding discerned the advantages of the production and consumption of cultured foods. Societies matured with the discoveries that these new products provided a source of nourishment more resistant to spoilage and less likely to cause food poisoning while increasing the seasonal availability of foods and offering new taste sensations. Even with the advent of new and improved food technologies involving production, harvesting, processing, packaging, and storage that reduce the importance of fermentation for food safety and shelf life, fermented foods and beverages continue to represent a major segment of the diet for people around the world.

The means by which fermented foods and beverages resist deterioration from spoilage microorganisms (and to some extent degradative enzymes) include the release of antimicrobial metabolites (eg, organic acids, bacteriocins, alcohols, fatty acids, enzymes) and alteration of the conditions in the food (eg, lowering of redox potential or water activity and competition or removal of nutrients and growth factors). In most cases, conditions are provided in the foodstuff to select for the fermenting microbiota to bring about desired changes. For example, the addition of salt and the maintenance of anaerobic conditions, as is the case for many fermented meats and vegetables, allows for the selection of various types of lactic acid bacteria that are originally present in the foods in relatively low numbers. With fermentable sugars present, and given the inherent ability of lactic acid bacteria to thrive in conditions of high salt concentration and anaerobiosis, the lactic acid bacteria flourish and the food is stabilized by a combination of acids produced by the lactic acid bacteria in accompaniment with the salt and other preservative compounds and conditions that pertain.

More recently, it has been established that some of the compounds produced by fermenting cultures can be extracted and purified for use as additives to preserve foods. Presently, consumer demand grows for foods minimally processed or reformulated to remove fat and salt while lowering calorie levels. In addition, there is a perceived advantage to "natural" foods or foods devoid of preservatives and chemical additives. One approach to deal with this trend while maintaining product safety, shelf life, and acceptance is to use "natural" by-products, extracts, or cultures of food-grade or GRAS (generally regarded as safe) microorganisms that have a long history of use and safety in fermented foods and beverages. New applications of such preservative agents should not significantly change the sensory characteristics of the food. Nisin, the bacteriocin produced by some strains of the cheese starter bacterium *Lactococcus lactis* subspecies *lactis*, has been the prime example of a microbial by-product that can be used successfully as a food preservative. This chapter will examine the characterization and use of bacteriocins for purposes of food preservation.

12.2 THE PRESERVATION OF FOODS INVOLVING TRADITIONAL METHODS OF FERMENTATION

Fermented foods and beverages are covered in several chapters in this book. This chapter will summarize the preservation mechanisms functioning in fermented products that reduce or prevent their spoilage and enhance the safety of the product, and overview the natural compounds that are produced by microorganisms and are active preservative agents.

12.2.1 *Saccharomyces cerevisiae* and the Killer Factor

Arguably, from a commercial standpoint the most important microorganisms in the production of fermented foods and beverages in the world are the fermentative yeasts, based on world consumption of alcoholic beverages and leavened breads. The most widely used of this group of microorganisms is *Saccharomyces cerevisiae*. This commercial importance has resulted in a wealth of scientific study on strains of *S. cerevisiae* both in the areas of basic yeast biology and applied starter culture technology.

From a food preservation standpoint, *S. cerevisiae* and other ethanologenic yeasts establish an environment nonconducive to growth of other microorganisms by excretion of large quantities of ethanol and carbon dioxide. Also, various acids are released as a result of the metabolism of sugars and significantly lower the pH. Ethanol has long been used as a disinfectant and represents one of the significant preservative components of alcoholic beverages.[208] The evolution of carbon dioxide from metabolizing strains of yeast also acts as an antimicrobial agent.[43,73] Conditions of anaerobiosis are generated in foods and beverages fermented by yeasts by the production of carbon dioxide along with the utilization of oxygen by the metabolizing yeast. Fermentative sugars, vitamins, minerals, and other nutrients are quickly utilized by metabolizing yeasts depriving these nutrients from competitive microbiota.

The levels of ethanol, carbon dioxide, acids, and other by-products from the metabolism and growth of yeast change the sensory quality of the product. As with nisin, an antibiotic substance from yeast that could act as a food additive for purposes of preservation would prove valuable if such a compound was safe and effective at low concentrations while imparting no significant changes in the aroma, taste, texture, and appearance of the product. Killer yeasts appear to have potential for the production of such preservative compounds.

Killer yeasts were first documented in 1963.[22] They are able to inhibit the growth of other yeasts by the production of toxins. Analogies can be made with bacteriocin production in bacteria.[174] Killer yeasts are resistant to the toxins they produce, and these toxins have a somewhat narrow target spectrum, effective only against specific types of other yeast. Molds, bacteria, and viruses are not affected. Killer toxins of yeast act to destabilize the integrity of the cellular membrane by forming and interfering with amino acid uptake[169] ion channels.[160,230] These extracellular toxins are proteins or glycoproteins with a molecular weight of approximately 32,000.[25,33] The binding of the killer toxin to the cell is by a specific receptor.[35] Just as contaminating nisin-positive lactococci slow or eliminate the activities of industrial cheese starter lactococci, so have contaminating killer yeast proved lethal to commercial ale and lager strains of yeasts.[163]

The killer and resistance factors in yeast are mediated by a double-stranded RNA (dsRNA) that resembles an intracellular virus.[208] Yeasts lacking this dsRNA are usually eliminated by the killer factor. Two forms of the dsRNA have been identified: MdsRNA and LdsRNA.[261] MdsRNA is found only in killer strains of *Saccharomyces* and encodes for the toxin and the immunity factor. Cells of killer strains harbor about 12 copies of the MdsRNA. LdsRNA is more common and has been found in both brewing and killer strains of yeast. Apparently its function is to encode for the capsid protein of the virus-like particle that surrounds the dsRNA. During cell replication, these particles are extracellularly maintained with some regulation from a chromosomal gene for expression of the killer phenotype.

The advantages of genetically modifying brewing strains with killer factors are that such cultures would be immune to the effects of contaminating wild killer yeast, and wild nonkiller yeast would be deterred from contaminating the fermentation by the killer brewing strain. This situation should theoretically help reduce the occurrence of faulty fermentations, although quality control/good manufacturing practices would represent the primary means of ensuring successful fermentations.

Killer toxins have been designated K1, K2, etc, with at least 10 types described, although only one toxin is produced for each strain of killer yeast.[208] Killer strains of *S. cerevisiae* are ineffective against *Candida* spp., *Pichia* spp., *Debaromyces* spp., *Kloeckera apiculata*, and several other yeasts. Commercial uses of this antagonistic mechanism have been inconsistent due in part to this somewhat limited spectrum of antagonistic action.

K1 toxin is the most highly characterized killer toxin with a molecular weight of 20,000.[26] It has α and ß subunits of 103 and 83 amino acids, respectively, linked by disulfide bonds. It appears the α subunit is responsible for the ion channel formation while the ß subunit binds to the cell wall receptor. A concentration of 2.3 ng ml^{-1} of K1 will kill approximately 2.0×10^7 cells of yeast ml^{-1}. The killing of the cells is an energy-dependent process that takes about 1 to 3 hours.[34] Macromolecular synthesis is followed by loss of K^+ ions and ATP with loss of amino acid uptake and cessation of proton pumping. As seen with many bacteriocins, cell lysis is not caused by the killer toxins.

Killer yeasts are generally only effective in the pH range of 4.0 to 5.4 and are ineffective at temperatures less than 30°C.[125] Such parameters limit the use of killer yeast in wine-making, although genetic modification could broaden the range of killer yeast effectiveness in these fermentations.

Yeasts are not necessarily limited to killer toxins as possible biopreservative agents. Suzzi et al.[240] examined 586 natural wine yeasts of different genera for antagonism to fungal pathogens isolated from fruits and soil. Fungal species representing *Aspergillus*, *Alternaria*, *Botrytis*, *Cladosporium*, *Fusarium*, and *Penicillium* among others were included. Two strains of *S. cerevisiae* and one of *Zygosaccharomyces* exhibited a broad spectrum of antagonistic activity against 10 fungal pathogens. McLaughlin et al.[167] found that *Candida* spp. displayed antagonism to a range of different fungal pathogens. Ben-Arie et al.[19] demonstrated that *Hanseniaspora uvarum* was effective against species of *Rhizopus* and *Botrytis*. Other researchers have found similarly effective growth inhibition of yeasts against molds.[166,210,262] The use of yeasts as effective biocontrol agents in fruits and vegetables has been suggested and continues to receive considerable attention[263,264]; however, there is no evidence of successful commercial adaptation.

12.2.2 Acidulating Bacteria

Organic Acids

As their name implies, the important unifying trait of the lactic acid bacteria is the production of organic acids (specifically lactate) from fermentable sugars. The lactic acid bacteria traditionally used in the production of acidulated, fermented foods or as probiotic additives are identified as GRAS organisms. However, there is concern that lactic acid bacteria occasionally can act as opportunistic or secondary pathogens and, in fact, the frequency of reports associating lactic acid bacteria with human clinical infection has been increasing.[1,100] It is well-known that some types of acidulating or lactic acid bacteria reside in the mouth and cause dental caries (eg, *Streptococcus mutans*, *Bifidobacterium dentium*), but these types are not used as starter cultures nor are they active in food fermentations. Of the food-associated lactic acid bacteria, the enterococci are probably the major focus of safety concern due to their intrinsic resistance to a wide range of antibiotics, presence in the human gut, and capability to transfer antibiotic resistance to pathogens.[1] In addition, enterococci have been isolated from human infections. Also of concern are the reports about members of the genus *Leuconostoc* responsible for generalized infections in adults, and some species of *Lactobacillus* associated with endocarditis and localized infections.[1] Therefore, even though food-related lactic acid bacteria have a well established, extensive record of safety and benefit, an awareness of their possible human pathogenicity is prudent. Certainly an *in vivo* assessment of the disease potential of lactic acid bacteria using improved modern methods of isolation and identification would be most valuable.

The means by which lactic acid bacteria deter undesirable microorganisms include the primary metabolites produced by carbohydrate catabolism (organic acids, carbon dioxide, alcohols, aldehydes, and ketones), hydrogen peroxide, diacetyl, and bacteriocins. Other types of antagonistic compounds (such as reuterin) have been documented as well, indicative of the rather complex antagonistic systems created by metabolizing lactic acid bacteria.[64]

Foremost in contributing to the preservative effect of lactic acid bacteria and other food-related fermenting bacteria, such as the propionibacteria and bifidobacteria, is the production of organic acids such as lactic, acetic, and formic acids. The drop in pH combined with the presence of these weak organic acids readily creates an environment hostile to many types of spoilage and pathogenic bacteria, as well as some fungi. The undissociated forms of the acids can pass through the cytoplasmic membrane and then dissociate intracellularly (see Chapter 8).[29] Acetate (pK_a at 25°C = 4.75) is a more effective inhibitive agent than lactate (pK_a at 25°C = 3.86) to some extent because of its higher dissociation constant.[70,112]

In lactic acid bacteria, ATP is synthesized by an extremely efficient carbohydrate fermentation coupled to substrate-level phosphorylation.[10] Under optimal conditions of growth, the homofermentative lactic acid bacteria (lactococci, pediococci, streptococci, and some lactobacilli) produce lactic acid almost exclusively from utilization of fermentable carbohydrates using the Embden-Meyerhof-Parnas pathway (glycolysis). Leuconostocs and heterofermentative lactobacilli use the hexose monophosphate shunt employing phosphoketolase. This pathway results in carbon dioxide and ethanol being produced in addition to lactic acid. Under nonoptimal growth conditions or by the utilization of certain substrates, homofermentative lactic acid bacteria employ the hexose monophosphate shunt that leads to end-products other than lactic acid.

Given the end-products produced during fermentation, bifidobacteria and propionibacteria could also be technically described as heterofermentative lactic acid bacteria; however, the pathways and end-products of these two microbial groups deviate from the hexose monophosphate shunt used by leuconostocs and heterofermentative lactobacilli. Bifidobacteria and propionibacteria are not closely related to the conventional lactic acid bacteria, but belong to the Actinomycetes. In *Bifidobacterium*, hexoses are degraded exclusively by the fructose-6-phosphate pathway that features fructose-6-phosphate phosphoketolase.[63,220] The acids from this pathway vary among the bifidobacteria. These primary metabolites are acetic and lactic acids with formic acid sometimes produced.[12] During the making of Swiss cheese, *Propionibacterium* convert lactate produced by lactic acid bacteria into carbon dioxide, propionate, acetate, and carbon dioxide.[106,149] The antimicrobial effectiveness of propionic and acetic acids are enhanced by the low pH found in fermented foods.[15]

Hydrogen Peroxide

Hydrogen peroxide is a highly reactive compound; in high concentrations it is quite toxic to biological systems. In aseptic processing, it is used to sterilize packaging containers. Lactobacilli, lactococci, leuconostocs, and pediococci are capable of producing hydrogen peroxide when grown in the presence of oxygen through electron transport by different mechanisms involving flavin enzymes.[49,139] Because lactic acid bacteria are catalase-negative, hydrogen peroxide can accumulate to high, autoinhibitory levels in the growth medium,[9] although the induction of stress proteins allows lactic acid bacteria to tolerate higher concentrations than other types of bacteria.[47] Bacteria such as pseudomonads and *Staphylococcus aureus* are up to 10 times more sensitive to hydrogen peroxide than lactic acid bacteria.[112] For example, evidence has been provided for the contribution of hydrogen peroxide in the inhibition of pseudomonads by *Lactobacillus plantarum*,[197] *L. delbrueckii* subspecies *bulgaricus*,[84] *L. delbrueckii* subspecies *lactis*,[159] *L. acidophilus*,[44] and lactococci.[136] Because lactic acid bacteria can produce a complex array of antimicrobial substances and create conditions that are unsatisfactory for spoilage and for the growth of pathogenic organisms, it is difficult to segregate the effectiveness of one factor from other factors, especially because the sensitivities of target microorganisms is often blurred by the combination and synergy of individual antagonistic factors.[64,85,200]

The Lactoperoxidase System

In raw milk, hydrogen peroxide also functions in the lactoperoxidase system.[13,209] Lactic acid bacteria contribute to the functioning of this system by generating hydrogen peroxide; starter cultures have been known to enhance the lactoperoxidase system. Notably effective against pseudomonads and other Gram-negative psychrotrophs, lactoperoxidase in raw milk catalyzes the oxidation of thiocyanate by hydrogen peroxide to form hypothiocyanite ions, higher oxyacids, and labile oxidation products.[81,196] Bovine milk naturally contains lactoperoxidase, thiocyanate, and hydrogen peroxide, but to make an effective preservation system, thiocyanate and hydrogen peroxide are often added (12 and 8 ppm, respectively). This system works optimally at 30°C and can extend the shelf life of raw milk from two days to five days. This makes the lactoperoxidase system a potentially important means of preserving milk in those parts of the world where refrigeration and use of pasteurization are limited.

Other Low Molecular Weight Metabolites

The fragrance and flavor of butter is delivered primarily by the compound diacetyl (2,3-butanedione).

Diacetyl, derived from the catabolism of citrate, is excreted by lactococci, pediococci, and leuconostocs. In products such as cream cheese and buttermilk, diacetyl is highly desirable, while in beer, even trace amounts are avoided. Diacetyl has been shown to have an antagonistic effect against a range of microorganisms including yeasts and molds as well as bacteria,[99,130,179,194] but its intense aroma and volatility severely limit its use in foods where the presence of diacetyl at effective, preservative levels (200 to 400 μg ml^{-1}) would be necessary.[53]

Reuterin is produced by *Lactobacillus reuteri* from the anaerobic catabolism of glycerol and glyceraldehyde.[11,39,242] This compound, not a bacteriocin, is an equilibrium mixture of monomeric, hydrated monomeric, and cyclic dimeric forms of ß-hydroxypropionaldehyde. Reuterin is strongly antagonistic against a wide range of Gram-negative and Gram-positive bacteria, yeasts, molds, and protozoa, probably because of the inhibition of ribonucleotide reductase.[243] The addition of reuterin to refrigerated ground beef effectively inhibits *Escherichia coli* and other microorganisms resulting in a shelf life extension compared with controls (Figure 12-1). When *L. reuteri* and glycerol are topically applied to herring, surface spoilage bacteria and accumulation of total volatile basic nitrogen are reduced during refrigerated storage under controlled atmospheres.[152] It appears that reuterin or reuterin-producing *L. reuteri* have the potential for use as biopreservative agents for refrigerated meats and seafood.

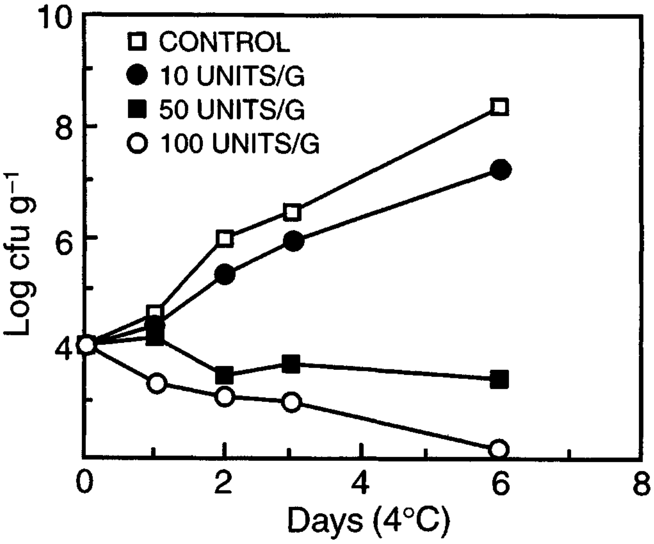

Figure 12-1 Effect of Reuterin on Coliforms in Ground Beef. *Source:* Adapted with permission from M.A. Daeschel, Antimicrobial Substances from Lactic Acid Bacteria for Use as Food Preservatives, *Food Technology,* Vol. 43, p. 226, © 1989, Institute of Food Technologists.

Microgard®

Microgard® is a commercial food additive that is a growth extract from *Propionibacterium freudenreichii* subspecies *shermanii.* Skim milk is fermented with the propionibacteria and then pasteurized to inactivate the culture.[254] The antagonistic effect of Microgard® is due to the metabolic by-products from the culture that includes acids, bacteriocins, and diacetyl.[7] It is marketed as a food preservative for use in such products as cottage cheese and fruit-flavored yogurt.[217] Microgard® has broad-spectrum activity against Gram-negative bacteria, yeasts, and some molds. Salih et al.[217] found Microgard® effective against Gram-negative psychrotrophic bacteria (Figure 12-2); the shelf life of cottage cheese was extended 6 to 9 days. Although the active components of the mixture have not been completely characterized, Al-Zoreky et al.[7] have identified a bacteriocin of 700 daltons that appears to generate considerable antimicrobial effect. Apparently, a newer version of Microgard®, which has nisin and other bacteriocins added, will soon be on the market. Given the relative success of Microgard® in the marketplace, other growth extracts using GRAS-status, acidulating bacteria should have commercial potential. For example, perhaps an extract of growth from a culture of *Acetobacter* spp. would have potential as a preservative with specific effectiveness against Gram-negative bacteria.

12.2.3 Protective Cultures and Probiotic Cultures

Protective Cultures

The term *protective culture* can be used to identify a strain or a mixture of strains added to a food for the primary purpose of improving safety of the product without changing the sensory characteristics of the food.[112] This distinguishes a protective culture from a starter culture, in that the starter culture is used to bring about intended compositional and sensory changes in a food (ie, a fermented food). Enhanced product preservation or an antimicrobial effect can be considered an associated benefit. Therefore, one culture can function as a starter culture or a protective culture depending on the allowed level of microbial activity in the food. As described above, Microgard® could be termed an example of a protective culture with the bacteria prevented from further metabolic activity by pasteurization. Pas-

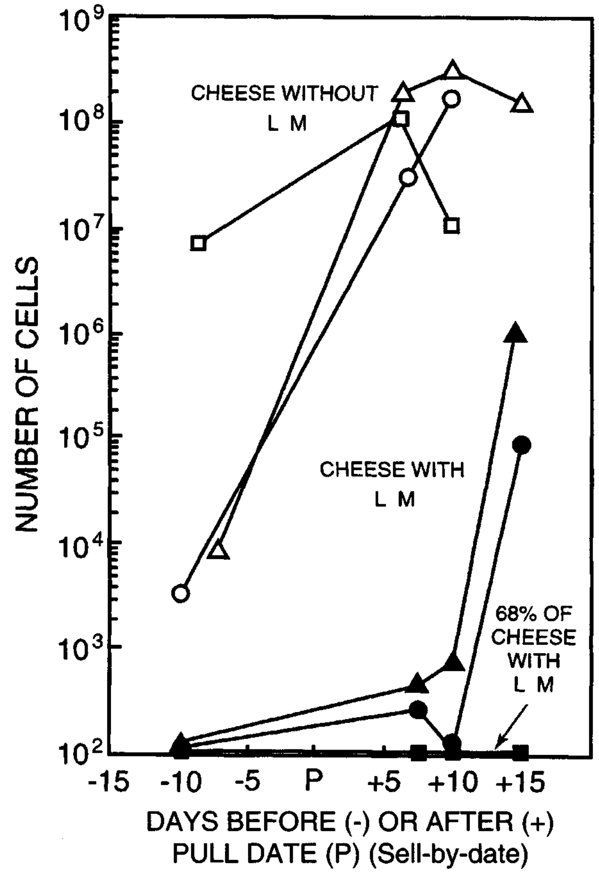

Figure 12-2 Inhibitory Effect of Liquid Microgard® (LM) against Spoilage Gram-negative Bacteria of Commercially Produced, Refrigerated Cottage Cheese. Open symbols represent three control groups without LM and closed symbols represent three test cheese groups with 1% LM. The cottage cheese was stored at 7°C for up to 30 days. *Source:* Reprinted with permission from M.A. Salih, W.E. Sandine, and J.W. Ayres, Inhibitory Effects of Microgard on Yogurt and Cottage Cheese Spoilage Organisms, *Journal of Dairy Science,* Vol. 73, pp. 887–893, © 1990, American Society of Animal Science.

teurization would not be a requirement for use of all protective cultures, but should the culture not be impaired or inactivated in some manner, a change in storage conditions of the product could result in revived culture metabolism in the food with possible deterioration of the sensory quality, such as over-acidification.

The Wisconsin Process for the preservation and safety of bacon with reduced levels of sodium nitrite employs a protective culture of *Pediococcus acidilactici.*[245] Sodium nitrite is an effective inhibitor of *Clostridium botulinum*; however, upon cooking, sodium nitrite in meat can form carcinogenic nitrosamines. The Wisconsin Process was devised to produce bacon as antibotulinal as regular bacon, but with lower levels of sodium nitrite. In this process, bacon is made with 40 to 80 ppm sodium nitrite, *P. acidilactici,* and 0.7% sucrose. This bacon has the same shelf life and sensory quality as regular bacon made with 120 ppm sodium nitrite.[244,245] If Wisconsin Process bacon is temperature abused, the protective culture grows and metabolizes the sugar to produce acid and other inhibitory compounds to prevent growth of, and toxin production by, *C. botulinum*. Excessive temperature abuse results in a soured but safe product.

Probiotic Cultures

Another category of microorganisms used as food additives is probiotic cultures. In this case, one can view the site of biocontrol as the human gastrointestinal tract. With the rise in consumer awareness of individual health, nutrition, and well-being, the interest and demand for value-added foods and beverages with enhanced nutritional benefit continues to expand. Rapid growth is anticipated in the American marketplace for cultures used as "nutriceuticals"; by 2010 industry analysts predict this market will grow about six-fold to $50 billion per year.[82] Terms such as nutriceuticals and functional foods are used to describe foods or components of foods that can prevent and treat diseases.[86] Use of these terms infers that in addition to its nutritive value, a functional food is consumed as part of the diet and not in supplement form (eg, capsule or tablet). It is a food derived from naturally occurring ingredients, and has a defined beneficial function when ingested. These functions may include the enhancement of the body's defense mechanism, prevention or recovery from a specific disease, the control of physical and mental conditions, and slowing of the aging process. Probiotic cultures are a category of nutriceuticals: viable cultures added to foods with the intent of maintaining or improving the nutritional health of the consumer.

The concept of ingesting viable, beneficial lactic acid bacteria in foods and beverages for improvement in general health and nutrition has existed since the turn of the century.[116] The major proponents were Elie Metchnikoff and his associates at the Institut Pasteur in the years before World War I. Currently, the favored groups for use as therapeutic human dietary adjuncts are the lactobacilli and bifidobacteria, although human probiotic preparations incorporating enterococci, propionibacteria, and *Saccharomyces* strains have been developed.[150]

Health benefits suggested from the regular ingestion of probiotic cultures are improvement of lactose digestion, treatment of diarrhea, serum cholesterol reduction, cancer suppression, immune system stimulation, treatment of constipation, and treatment of vaginitis.[219] Additional benefits are deconjugation of bile salts, stimulation of peristaltic movement, control of mucin at the intestinal surface, metabolism of ammonium ions, aid in absorption of calcium, and synthesis of vitamins.[114] Exhibit 12-1 lists these suggested benefits and others. Many opinions exist as to the general effectiveness of lactic acid bacteria and other acidulating bacteria in the promotion of human health. Further clarification is required for many of these claims to achieve wide acceptance by regulatory agencies and the general public.

One of the indicated benefits of regularly ingesting probiotic cultures is the antagonistic action against acid-sensitive enteric pathogens with maintenance of a lower fecal pH and a more stable equilibrium of the intestinal microbiota. Production of antimicrobial products by probiotic cultures represents a mechanism for maintaining a healthy gut equilibrium and is one of the desired properties for a good probiotic strain.[78,88,218] Although the mode of action for a positive probiotic effect is very complex and is often unclear, the mechanisms in which probiotic cultures are assumed to act are competition versus other bacteria for nutrients and for adhesion receptors on the gut epithelium and stimulation of immunity and production of antibacterial substances.[79] Therefore, in addition to the production of antimicrobial substances and conditions effective against detrimental gut bacteria, other desirable strain characteristics involve culture maintenance and viability, and consumer safety. These traits would be represented by good *in vitro* growth, minimal loss of culture viability during product storage, stability after exposure to acids and bile salts, and safety for human use that usually implies toxicological studies or long-recognized GRAS status and original isolation and abundance in the human intestinal tract.[218]

Exhibit 12-1 Suggested Benefits of Ingesting Bifidobacteria and Other Probiotic Cultures

- Stabilization of intestinal microbiota
- Induce cell-mediated immunity
- Anti-tumorigenic
- Reduce serum cholesterol
- Aid digestion, especially of lactose
- Deconjugation of bile salts
- Stimulate peristaltic movement
- Control mucin at intestinal surface
- Degrade nitrosamines
- Metabolize ammonium ions
- Aid in absorption of calcium
- Vitamin synthesis
- A key to infant nutrition
- Treatment of some diarrheas
- Intestinal recolonization following antibiotic treatment, chemotherapy, or radiation treatment
- Prevent vaginal yeast infections

Renewed interest in probiotic application has caused more intense study of the bifidobacteria. Once identified as lactobacilli, bifidobacteria continue to draw attention as human probiotics due in part to the production of acetic and lactic acids from fermentable sugars and their natural predominance in the human colon. However, probably more of a basis for selection of bifidobacteria for development as probiotic agents has been the early documentation of high levels of bifidobacteria in fecal samples from breast-fed infants as compared to weaned or bottle-fed infants.

Bifidobacteria can be isolated from feces of humans at any age. At birth, bifidobacteria are one of the first groups to establish themselves in the intestinal tract and usually are the largest group represented in infants. For breast-fed babies, levels of 10^{10} to 10^{11} per g of feces are common.[171] Bifidobacteria constitute over 25% of the intestinal flora in healthy breast-fed infants, while lactococci, enterococci, and coliforms represent less than 1% of the population, and bacteroides, clostridia, and other organisms are absent.

In comparison, bottle-fed babies normally have 1-$\log_{10}$ count less bifidobacteria present in fecal samples than breast-fed babies, and bottle-fed infants generally have higher levels of Enterobacteriaceae, streptococci, and anaerobes other than bifidobacteria.[171] With the change of diet and the aging process following infancy, the level of bifidobacteria declines so that Bacteroidaceae predominate in the adult gut, with eubacteria, bifidobacteria, and Peptococcaceae represented in that order. The superiority in overall health of breast-fed infants compared with bottle-fed infants is fairly well established, therefore the associated findings of high numbers of acidulating bifidobacteria relative to other bacteria in the gut imply a contribution of *Bifidobacterium* to this state. Results from fecal counts are often affected by the type and number of volunteers contributing fecal samples, diet, and sample analysis and interpretation.

Another approach to attain elevated levels of gut bifidobacteria is the ingestion of prebiotic substances (with or without probiotic cultures).[83] Such prebiotic compounds are bifidogenic factors that stimulate proliferation of resident bifidobacteria while the growth of undesirable bacteria, such as clostridia, is not enhanced. In the 1950s, it was commonly believed that human milk was a requirement for the growth of bifidobacteria. It is now established that a range of compounds can act as bifidogenic substances and the early emphasis on mother's milk as a source of bifidogenic substances was overestimated. In *Bifidobacterium*, growth requirements and growth-promoting factors vary from species to species. Most of the bifidogenic compounds identified are amino sugars that occur in human colostrum or other natural sources.[173] For some species of *Bifidobacterium*, bovine casein digest and yeast extract have been reported to have a more consistent growth-promoting effect on bifidobacteria than human milk whey.[195] Other examples of bifidogenic compounds are lactoferrin, lactulose, lactitol, and oligosaccharides.[12] Fructo-oligosaccharides have received much attention, especially in Japan where they have become a common food additive.[172] Bifidogenic fructooligosaccharides such as inulin can be obtained from Jerusalem artichokes, chicory, and Dahlia roots.

Bifidobacteria and lactic acid bacteria can be used clinically to treat diarrheal diseases and intestinal infections, especially in the very young and very old.[46,121,171] For example, Saavedra et al.[215] have presented strong evidence for the prevention of diarrhea and shedding of rotavirus by feeding infants a milk formula supplemented with *Bifidobacterium bifidum* and *Streptococcus thermophilus*. The double-blind, placebo-controlled study not only demonstrated a reduction in the incidence of acute diarrhea and rotavirus shedding in 55 subjects, but the probiotic formula was also well tolerated by the children, many of whom were initially malnourished or immunocompromised because of underlying disease. In a similar Japanese study with a powder containing 10^9 to 10^{10} g^{-1} of *Lactobacillus casei*, favorable results were indicated from a subject pool of 32 infants ($\leq$1-year-old) suffering from rotavirus enteritis.[239] These data suggest probiotic microbial preparations may provide a practical method for the prevention of diarrheal disease in a wide range of clinical and environmental conditions.

Other clinical trials have involved the influence of probiotic feeding on the metabolic (ie, enzymatic) activity of the resident microbiota. A significant reduction in the fecal activities of bacterial ß-glucuronidase, azoreductase, and nitroreductase was found with the administration of *Lactobacillus acidophilus* isolated from humans.[87] In another human feeding study,[158] fermented dairy products containing high numbers of *B. bifidum* and *L. acidophilus* caused a substantial decline in fecal nitroreductase activity, however, no significant decrease in ß-glucuronidase or azoreductase activity occurred. The yogurt cultures, *S. thermophilus* and *L. bulgaricus*, have been shown in a human feeding study[191] to have no effect on fecal enzyme activities; however, ingestion of *Lactobacillus gasseri* for 12 days dramatically decreased all three enzyme activities in fecal samples. Such measurement of fecal enzyme activities give an approximation of the alteration of intestinal metabolism in a beneficial manner. Clearly, this effectiveness is dependent upon the type of probiotic cultures used. Several probiotic preparations decrease fecal enzyme activity[150] and imply a decrease in the mutagenicity of the intestinal contents.[151]

In the last 10 years or so, a significant momentum of activity has occurred in the study of bacteriocins from lactic acid bacteria and other acidulating GRAS bacteria that have potential application in foods and beverages as "natural" preservatives. The information generated in the study of food-grade bacteriocins can potentially be used to genetically improve probiotic cultures in an effort to improve the antimicrobial nature of these strains in the human gut. Conversely, bacteriocins discovered from clinical isolates of probiotic cultures have the potential for development as commercial food preservatives.

12.3 BACTERIOCINS

12.3.1 History and Microbial Ecology

The earliest workers in microbiology recorded antagonism between competing microorganisms.[113] In 1676, Antonie van Leeuwenhoek first documented antibiosis, observing that the product from one microorganism inhibited the growth of another. In 1877, Pasteur and Joubert recorded the inhibition of common bacteria from urine against *Bacillus anthracis*. In the 20th century, the mechanisms for these events began to be revealed and antibiotic substances, including antibacterial peptides and proteins, were shown to play an important role in microbial interaction and competition.

Originally the term "bacteriocin" developed with emerging information from study of the colicins, which were the prototype bacteriocins. Their activity was first described by Gratia in 1925; "principe V" was produced by one strain of *Escherichia coli*

Next Page

against another strain of *E. coli*. The term "colicine" was coined by Gratia and Fredericq in 1946.[164] Later, "bacteriocine" was used by Jacob et al.[124] as a general term for highly specific antibacterial proteins. The term colicin now implies a bactericidal protein produced by varieties of *E. coli* and closely related Enterobacteriaceae.[146] Therefore, bacteriocins were originally defined as bactericidal proteins characterized by lethal biosynthesis, a very narrow range of activity, and adsorption to specific cell envelope receptors.[124] Following further study, a later amendment was the association of bacteriocin biosynthesis with plasmids.[241] While there is no universally accepted definition, the term has now been generalized and expanded to encompass the differences between colicins and bacteriocins produced by Gram-positive bacteria. Today, all antibacterial peptides and proteins produced by bacteria are referred to as bacteriocins. In addition, a broader range of target bacteria is now incorporated into the definition along with the allowance that the peptides and proteins can contain other functional moieties, such as carbohydrates or lipids.[71,131]

Bacteriocin-producing bacteria can be isolated from a wide range of habitats; most if not all Gram-positive and Gram-negative bacteria produce bacteriocins.[216] It has been estimated that 40% of all strains of *E. coli* produce a colicin.[113] Given this commonality, it would appear bacteriocins offer bacteria an added adaptability or competitive advantage against other bacteria for location, food, and other resources.[71] It has been shown that bacteriocin-producing lactobacilli will persist longer and outcompete nonbacteriocin-producing lactobacilli in olive fermentations.[214]

In lactic acid bacteria as well as many other types of bacteria, depletion of the transmembrane proton motive force is the common mechanism of bactericidal and bacteristatic activities by bacteriocins.[24,30,31,40] In an intact, functioning membrane, there is a net proton gradient with a proton flow out of the cell, which causes the outer side of the membrane to have a positive charge and the inner side to have a negative charge. It appears that most bacteriocins form pores or ion channels in the cytoplasmic membrane resulting in loss of ions and the collapse of the proton gradient; this disrupts the generation of energy and causes death, injury, or inhibition of growth. Although this mode of action among bacteriocins appears widespread in nature, there is a broad range of molecular weights representing a divergent evolutionary response to the diversity of competing species.[71] While some colicins can form ion channels in the cytoplasmic membrane, colicins E2 and E3 and cloacin DF13 are enzymes; E3 and DF13 have RNase activity and E2 has restriction endonuclease activity.[154,199]

With the keen interest in developing bacteriocins as natural food preservatives for commercial application, the practical concern has arisen about the concurrent development of spontaneous bacteriocin resistance similar to that seen with the proliferation of antibiotic-resistant bacteria in response to increased use of therapeutic antibiotics. Populations of bacteriocin-sensitive bacteria will naturally contain bacteriocin-resistant varieties.[170] Presently, it is not certain whether immunity to bacteriocins will spread in the same manner and timetable as has immunity to commonly used therapeutic antibiotics.

Over the past several years, the increased interest in bacteriocins has generated a number of valuable review papers, books, and book chapters written to describe and review activities of the bacteriocins or of the antimicrobial action of bacteria with relationship to foods.[65,98,115,123,142,143,146,203,204,241] Exhibit 12-2 consolidates most of the key reasons for recent interest in bacteriocins produced by lactic acid bacteria.

Exhibit 12-2 Events Contributing to the Increasing Number of Applied Investigations on Bacteriocins from Lactic Acid Bacteria and Bacteriocinogenic Lactic Acid Bacteria

- Safe and efficacious use of nisin during the past 30 years.
- Food and Drug Administration (FDA) approval of nisin as a GRAS substance in certain applications.
- Consumer resistance to traditional "chemical" preservatives and a desire for "natural" preservation.
- Justifiable concerns over the safety of existing food preservatives such as nitrates and sulfites.
- Realization that bacteriocinogenicity is not a rare occurrence in lactic acid bacteria.
- Feasibility of using bacteriocin production and immunity as selectable genetic markers in starter culture bacteria.
- Availability of molecular biology techniques to transfer, clone, and sequence the genetic determinants and to engineer genetic determinants of bacteriocins.
- Willingness of federal funding agencies, food commodity groups, and food processing corporations to fund both basic and applied research.

Source: Reprinted with permission from M.A. Daeschel, Applications and Interactions of Bacteriocins from Lactic Acid Bacteria, in *Bacteriocins of Lactic Acid Bacteria,* D.G. Hoover and L.R. Steenson, eds., pp. 63–91, © 1993, Academic Press, Inc.

The bacteriocins from lactic acid bacteria are now categorized into four classes[143] (Exhibit 12-3). Lantibiotics (class I) are small membrane-active proteins that contain lanthionine amino acids (hence their name). Nisin is an example; subtilin from *Bacillus subtilis* has a common ancestry with nisin and is also a lantibiotic. Class I can be subdivided into subtypes A and B.[137] Nisin and subtilin are included in subtype A; this group features amphiphilic polypeptides, which are elongated and cationic, and act by disruption of proton motive force at the cytoplasmic membrane. Examples of subtype B are duramycin and cinnamycin. These bacteriocins are neutral, globular, amphiphilic polypeptides acting as enzyme inhibitors and immunoadjuvants. Class II bacteriocins from lactic acid bacteria include small heat-stable membrane-active nonlanthionine peptides. Leucocin A is a representative bacteriocin. Large heat-labile proteins such as helveticin J constitute class III. Plantaricin S is an example of class IV, containing complex bacteriocins with carbohydrate or lipid moieties.

12.3.2 Nisin

Nisin remains the current focal point by which all bacteriocins intended for use as biopreservatives are compared for potential commercial and regulatory acceptance. This ribosomally synthesized peptide has an established track record of performance as a food additive on the world market, and therefore has accumulated the greatest amount of practical information. Nisin has a documented record of safety in foods.[118] The activity of nisin was first observed in 1928.[211,212] It was named by Mattick and Hirsch[162] from "N inhibitory substance," because the industrial cheese starter cultures, group N dairy streptococci (now *Lactococcus lactis*) not only produced nisin, but nisin-negative strains were inhibited by it. Nisin was first used as a food preservative in 1951,[109] and in 1969, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives gave nisin international acceptance.[260] In the United States (US), it is presently the only bacteriocin permitted for use in foods as a food additive.[74] While other bacteriocins from lactic acid bacteria are receiving attention in the search for other applicable GRAS biopreservatives, nisin remains the preeminent model system for these types of compounds.

Exhibit 12-3 Classes of Bacteriocins from Lactic Acid Bacteria

Class I—Lantibiotics (heat stable)
Examples: nisin, lactocin S, lacticin 481, carnocin UI49

Class II—Small (<15,000 Da) heat-stable nonlantibiotic peptides
Examples: leucocin A, diplococcin, lactacins B and F, lactocin 27, lactococcin A, sakacins A and P, curvacin A, enterocins E1A and 1146, plantaricin SA6, pediocins, carnobacteriocins

Class III—Large (>15,000 Da) heat-sensitive proteins
Examples: helveticins J and V-1829, lacticins A and B, caseicin 80, acidophilucin A

Class IV—Complex bacteriocins with nonproteinaceous components
Examples: plantaricin S, leuconocin S

Source: Reprinted with permission from T.R. Klaenhammer, Genetics of Bacteriocins Produced by Lactic Acid Bacteria, *FEMS Microbiology Review,* Vol. 12, pp. 39–86, © 1993, Elsevier Applied Science.

There are several natural variants of nisin, all with 34 amino acids. The size of the pentacyclic monomeric form of nisin is approximately 3,500 daltons with dimer and tetramer forms common.[119] As noted earlier, nisin is grouped in the class of bacteriocins known as lantibiotics; besides subtilin,[126] other examples of lantibiotics are epidermin,[5] gallidermin,[140] cinnamycin, and duramycin.[92] The composition of nisin features the unusual amino acids, dehydroalanine, dehydrobutyrine, lanthionine, and ß-methyllanthionine[93] (Figure 12-3); however, peptide antibiotics that contain unusual amino acids are common.[95,96] Gramicidin S and valinomycin are two such examples that are synthesized by nonribosomal mechanisms, whereas nisin undergoes a novel sequence of posttranslational modifications of ordinary amino acids.[120] The sequence of the gene encoding for nisin production and its flanking regions have been elucidated.[32,68,138]

A majority of the genes for bacteriocins from Gram-positive bacteria are linked to plasmids. In the case of *nisA*, the structural gene for the nisin precursor has been associated with a 70-kb conjugative transposon.[69,202] Siegers and Entian[228] identified three open reading frames belonging to the nisin gene cluster of *L. lactis* 6F3, which also appear to be involved in the immunity of the nisin-producing strain to nisin. In this strain, the complete gene cluster for nisin biosynthesis is 15 kb. The gene *nisG* encodes for a hydrophobic protein, which probably acts similarly to the immunity proteins already described for several colicins.

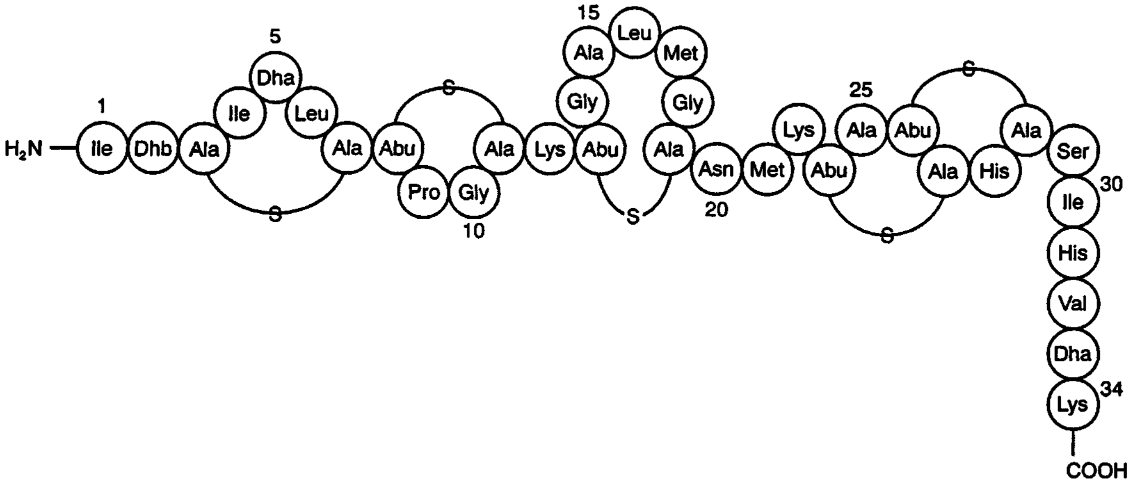

Figure 12-3 Nisin A. *Source:* Reprinted with permission from D.G. Hoover, Bacteriocins: Activities and Applications, *Encyclopedia of Microbiology,* Vol. 1, pp. 181–190, © 1992, Academic Press, Inc.

The techniques of protein engineering have been applied to nisin. Two mutants of nisin Z (a natural nisin variant) were found to have similar antimicrobial activity, but improved solubility at neutral pH and stability to pH and temperature.[213] In the nisin mutants, a lysyl residue replaced either an asparagine and a histidine in the primary sequence of nisin Z.

Target bacteria for the action of nisin include most Gram-positive genera, although the sensitivity of Gram-positive bacteria varies. Nisin-negative lactococci are susceptible as well as other lactic acid bacteria. Nisin-sensitive genera that contain important foodborne pathogens are *Listeria, Staphylococcus, Clostridium,* and *Bacillus.* Normally, Gram-negative bacteria are not sensitive to nisin; however, if the outer lipopolysaccharide layer is removed or absent from the cell boundary, the cytoplasmic membrane is no longer protected from the effects of nisin and such Gram-negative bacteria become nisin sensitive.[234,235] Compared with bacteriocins from other lactic acid bacteria, nisin has a rather broad spectrum of activity; however, when compared with therapeutic antibiotics, the range of activity of nisin is quite small. Nisin is not inhibitory against yeasts and molds.

Nisin is also quite effective against the spores of Gram-positive bacteria. This is a valuable characteristic because the endospores of Gram-positive bacteria are so resistant to heat and other methods used to process and preserve foods. In the US, nisin was approved for use in pasteurized, processed cheese spreads because of its effectiveness against gas-forming spoilage clostridia (ie, "blowing" faults caused by *Clostridium butyricum* and *C. tyrobutyricum*). Just as vegetative cells vary in sensitivity to nisin, so do bacterial spores. The spores of the small-spored species of *Bacillus* (eg, *B. subtilis*) are more sensitive to nisin than those of the large-spored species (eg, *B. cereus*).[90]

Spores of *C. sporogenes, C. butyricum,* and *C. bifermentans* have been shown to be much more sensitive to nisin than the respective vegetative cells.[108] Against *C. botulinum,* nisin is a more effective inhibitor of type E than type A; the range of nisin concentrations necessary to inhibit outgrowth from spores of *C. botulinum* is from 100 to 5,000 IU and in addition to strain type inhibition is dependent upon spore concentration and the medium or food examined.[225,226] To prevent spore outgrowth, it has been suggested that nisin covalently modifies the sulfhydryl groups of proteins of newly germinated spores.[97,175]

Nisin has been evaluated in a variety of foods and beverages; sometimes a valuable preservative effect is evident, but sometimes there is limited or no observable benefit.[66] For the antibiotic effect of nisin to be realized, nisin must be added to the product as a food additive or growth extract, or a nisin-positive culture of *Lactococcus lactis* subspecies *lactis* must actively grow in the product, as in the production of a fermented food. No nisin synthesis takes place during the stationary phase of the producing culture.[117]

Probably the greatest application for nisin as a food preservative is in milk and dairy products. This is only fitting given the commercial use of lactococci in cheese-making. Indeed, one of the early reports about inhibitory streptococci involved milk in which cheese starter organisms failed to develop.[259]

Many types of cheeses are susceptible to clostridial problems; Hirsch et al.[109] first used a nisin-positive starter culture in the making of Gruyère cheese to prevent undesirable clostridia from forming gas and butyric acid defects. Hawley[102] was the first to suggest that nisin be added to cheese milk as a mixture with powdered skim milk, a common practice today. The commercial preparation, Nisaplin®, is marketed in powdered form containing 2% active nisin with the remainder inert ingredients, notably nonfat dry milk and sodium chloride. To prevent significant loss in the whey, it is recommended that nisin be added after the lactic fermentation at concentrations of 40 to 400 IU g^{-1}. Of course, using nisin in this manner requires a nisin-resistant culture or a nisin-producing culture immune to the effects of nisin.[153] Another concern in using nisin is that nisin can interfere in the aging of cheeses by inhibiting bacteria important in flavor development. With the development of genetically engineered cheese starter cultures, it would seem that modifying industrial lactococcal strains for nisin synthesis and immunity along with proteinase activity for flavor development offers great potential for extended use of nisin.[27,77] This technology may be applied to other bacteriocins and culture characteristics as well.

Since the 1950s, nisin has worked well as a food additive in processed cheese products.[66,233] The addition of water and flavor compounds with heated mixing of the unripened curd allows survival and outgrowth of clostridial spores. Because no fermentation or late microbial flavor development occurs in the manufacture of processed cheese products, the disadvantages described above for ripened cheeses are absent. Levels of 100 to 250 ppm are normally used in these products. Somers and Taylor[233] examined the effectiveness of nisin in pasteurized process cheese spreads and found that in a cheese spread having a moisture content of 52% with 2.5% disodium phosphate but no added sodium chloride, 12.5 ppm nisin prevented outgrowth and toxin production by spores of *C. botulinum* (Table 12-1). It was suggested that the addition of nisin to cheese spreads (100 ppm or greater) can generate an equivalent antibotulinal effect while allowing for reduced sodium levels or somewhat higher moisture content compared with typical commercial pasteurized process cheese spreads.

Table 12-1 Toxicity Results on Process Cheese Spreads

% Na_2HPO_4	*Nisin (ppm)*	*Toxic Samples/ Total Sample #**
2.0	0	80/80
2.5	0	1/80
2.5	12.5	0/80
2.0	12.5	58/80
1.7	0	80/80
1.7	12.5	10/80
1.7	50	1/80
1.7	100	0/80

*Pasturized process cheese spreads were inoculated with approximately 10^3 g^{-1} spores of *Clostridium botulinum* and incubated at 30°C for up to 48 weeks. Toxicity was determined by the mouse injection assay.

Source: Reprinted with permission from E.B. Somers and S.L. Taylor, Antibotulinal Effectiveness of Nisin in Pasteurized Process Cheese Spreads, *Journal of Food Protection*, Vol. 50, pp. 842–848, © 1981, International Association of Milk, Food and Environmental Sanitarians, Inc.

In canned foods, nisin has been shown to increase the apparent heat sensitivity of bacterial endospores and to increase the efficiency of thermal processing, allowing for shorter times and lower temperatures to be used to attain commercial sterility.[37,38] Kiss et al.[141] showed in a pilot-plant study that 200 IU nisin ml^{-1} added to peas in jars permitted about a 60% reduction in the heat process resulting in an improved sensory quality of the peas. In those parts of the world where energy use is inconsistent or costly, the savings derived from the addition of nisin to low-acid canned foods for reduction of the energy costs of thermal processing offers a satisfactory return on the cost of nisin. It should be emphasized here that nisin is not employed in an antibotulinal role, but as an anti-thermophile agent for acid foods or for neutral-pH foods given a full antibotulinal cook.

Nisin at levels of 50 to 100 IU g^{-1} has been shown to be effective for a range of low-acid canned foods, such as vegetables, meats, seafood, mushrooms, dairy, and pasta products[76,153]; however, if the microbial quality of the product is poor, nisin will not mask deficiencies in good manufacturing practices. For example, even at high levels of 500 IU nisin g^{-1}, canned products such as soup, cream-style corn, and chicken chow mein will spoil if contaminated with heavy bacterial loads.[103,104,129]

In high-acid canned foods of pH below 4.5–4.6, more acid-tolerant, thermophilic spoilage spore formers are the target in exploiting the synergy of nisin use with thermal processing. In these foods, the lower pH improves the solubility, stability, and activity of nisin. Against spoilage bacteria, such as *Bacillus coagulans* and *Clostridium thermosaccharolyticum*, nisin concentrations of 50 to 200 IU g^{-1} can prevent spoilage in beans packed in tomato sauce, canned tomato products, and canned fruits.[38,62,104,252]

The results of using nisin and other bacteriocins in meats have not been very successful. The solid nature and composition of meats cause uneven distribution and binding or "neutralization" of nisin. At the neutral pH of most meat products, the solubility and stability of nisin is low, limiting its effectiveness. These factors result in an inconsistent preservative action of nisin against Gram-positive food pathogens and spoilage agents, even at extremely high levels of nisin. Against spores of proteolytic strains of *C. botulinum*, nisin concentrations from 1,000 to 5,000 IU g^{-1} were ineffective, even when used in combination with low levels of nitrite and mild acidification.[207,232] Similar results were obtained versus other spore formers and nisin-resistant lactic acid bacteria.[18,36,128] A disadvantage in using nisin for extension of the shelf life of meat products is that the metabolism and growth of psychrotrophic and mesophilic Gram-negative bacteria play an important role in spoilage of these products, but nisin alone has little effect on these bacteria. To deal with Gram-negative bacteria, Shefet et al.[227] combined nisin with food-grade chelating agents (EDTA, citric acid, and Tween 20) to extend the shelf life of broilers stored under refrigeration. *Salmonella typhimurium* inoculated onto fresh broiler drumstick skin was reduced by 3- to 5-$\log_{10}$ after a 30-minute immersion in a solution of nisin and chelating agents.

Recently, Einarsson and Lauzon[72] compared nisin Z with two other bacteriocins, carnocin UI49 (from *Carnobacterium piscicola* UI49) and bavaricin A (from *Lactobacillus bavaricus* MI 401). Nisin Z was more effective than the other two bacteriocins in the preservation of chilled brined shrimp, extending the shelf life of the shrimp 31 days beyond the preservative-free control. Carnocin UI49 showed no preservative effect, while bavaricin A extended the shelf life 16 days. However, 0.1% benzoate-sorbate solution preserved the brined shrimp for the entire storage period of 59 days.

In some cases, nisin is effective as an adjunct when combined with nitrite and other preservative agents or treatments[206,207]; however, in all these adaptations incorporating nisin, the cost of nisin plays a very important role in its commercial acceptance and use. Currently, chemical preservatives and mild heat treatments for meat products can usually be applied as effectively and more cheaply than nisin alone or as a processing aid. Under these circumstances, nisin often cannot compete economically. Future use of nisin and other bacteriocins will depend heavily on consumer reaction to the adjective "natural" in product labeling.

Usually the spoilage bacteria targeted for inactivation by nisin are the Gram-positive spore formers and the lactic acid bacteria. Lactic acid bacteria are sometimes a problem in the manufacture and preservation of alcoholic beverages. In wines, nisin can be used at 100 to 1,000 IU ml^{-1} to regulate uncontrolled malolactic fermentation caused by nonstarter pediococci, leuconostocs, and lactobacilli as well as other spoilage defects caused by lactic acid bacteria. Daeschel et al.[51] used nisin-resistant varieties of *Leuconostoc oenos* with 100 IU nisin ml^{-1} to regulate the malolactic fermentation in wine. In the maintenance of brewers' yeast, the use of nisin to wash the pitching yeast to rid it of unwanted lactic acid bacteria is recommended over the traditional harsher method of acid washing.[185] The nisin treatment effectively eliminates lactobacilli from the yeast starter culture, but leaves the *Saccharomyces* with improved viability and unchanged fermentative performance. Because *Saccharomyces* is unaffected by nisin, this can be added to the wort to prevent contaminated fermentations caused by lactic acid bacteria that produce off-flavors and other defects.[186] Nisin survives pasteurization, filtration, and fining in the making of malted beverages, and can be added after the yeast fermentation as a means of maintaining quality and enhancing flavor by allowing a reduction of heat treatment.

12.3.3 Bacteriocins Produced by Acidulating Gram-positive GRAS Bacteria

Other Bacteriocins Produced by Lactococci

Because the bulk of literature and product development concerns nisin, other bacteriocins produced by lactococci are sometimes overlooked. Contributing to this may be that diplococcin also has a long history (as far as bacteriocins are concerned), but has several inherent characteristics that severely curtail its development as a biopreservative. Produced by a minority of strains of *Lactococcus lactis* subspecies *cremoris*, diplococcin becomes very unstable when purified, has a limited spectrum of antagonistic activity (eg, it is ineffective against spore formers), and has a relatively high degree of sensitivity to trypsin, pronase, α-chymotrypsin, and most importantly, heat.[54,187] Initially, one might expect that diplococcin would be very similar to nisin because these bacteriocins are produced by two closely related subspecies of lactococci with demonstrated effects against other lactococci. Such is not the case; nisin is more closely related to subtilin produced by *B. subtilis*. Diplococcin is larger in size (5,300 daltons) and is not a lantibiotic; it does not contain lanthionine, ß-methyllanthionine, dehydroalanine, or dehydrobutyrine.[57] Instead of having potential commercial benefit in foods, diplococcin is a problem in that cheese-makers must be aware of diplococcin-producing lactococci that can rapidly predominate over other lactococci used in multiple-strain starter cultures.[56] In *L. lactis* subspecies *cremoris*, Davey[55] characterized an 81-kb conjugative plasmid harboring diplococcin synthesis and immunity, while Neve et al.[183] demonstrated conjugation of the ability to produce diplococcin with a 60-kb plasmid.

Another lantibiotic produced by *L. lactis* subspecies *lactis* that has been characterized is lacticin 481, a highly hydrophobic polypeptide of 23 amino acids.[192] Lacticin 481 is more closely related to streptococcin A-FF22 produced by *Streptococcus pyogenes* FF22 than nisin.[122,193] Although exhibiting a narrower spectrum of activity than nisin, lacticin 481 may have application as a preservative in cheese products as it has shown strong activity against *Clostridium tyrobutyricum*, is resistant to heat and rennet, and, unlike nisin, is stable at neutral pH.[192]

Nearly all *L. lactis* subspecies *diacetylactis* strains and some *L. lactis* subspecies *cremoris* and nonnisin-producing *L. lactis* subspecies *lactis* strains produce bacteriocins referred to as lactostrepcins.[147] Antagonism has been demonstrated against other lactococci, streptococci (groups A, C, and G), *Bacillus cereus*, and some *Lactobacillus* and *Leuconostoc*.[265] Lactostrepcins are group I bacteriocins that are heat stable (121°C for 10 minutes) and approximately 10,000 daltons. A pH ≤ 5.0 is required for optimal activity, but their effectiveness is limited because of their sensitivity to proteolytic enzymes, phospholipases, and lipases.[14,67]

Lactococcins is a term that can be used to encompass all the bacteriocins produced by *Lactococcus*: nisin, diplococcin, lacticin 481, the lactostrepcins and others, although in some cases the term is directly applied to the less characterized lactococcal bacteriocins eg, lactococcins A, B, and M. Different strains of the three lactococcal subspecies can produce the same lactococcins, although the plasmids encoding the bacteriocins display different sizes and profiles among these strains.[111,221,249,250] Holo et al.[111] has purified and characterized lactococcin A, a small, hydrophobic polypeptide produced by *L. lactis* subspecies *cremoris* LMG 2130 that is effective only against other lactococci.

Lactobacilli

The lactobacilli are a very heterogeneous group composed of a rich diversity of related microorganisms. Early work with specific bacteriocin activity in *Lactobacillus* was done by De Klerk and coworkers,[59–61] although the general inhibitory effect of the growth of lactobacilli was studied in the late 1940s with dairy lactobacilli.[94,253,258] Bacteriocin activity was suggested during this period, lactobacillin from *Lactobacillus delbrueckii* subspecies *lactis* was named by Wheater et al.[256,257]; however, as discussed earlier, it can be difficult to separate bacteriocin activity from the other antibacterial substances and conditions produced by lactic acid bacteria. Throughout the 1970s, the antagonistic activities of lactobacilli were often poorly characterized. Inhibition by lactobacilli was commonly demonstrated against a wide array of Gram-positive and Gram-negative bacteria; some inhibition could be eliminated or reduced by adjustment to neutral pH or by treatment with catalase, indicating that organic acids and hydrogen peroxide made significant contributions to the inhibition. Most bacteriocins from lactobacilli inhibit only other lactobacilli.

Since the 1980s, Klaenhammer and coworkers have closely examined the genetics and biochemistry of several bacteriocins from *Lactobacillus*. Lactacin B is produced by *L. acidophilus* N2.[16] Lactacin B forms aggregates; the molecular weight of the monomer is 6,000 to 6,500 and aggregates are as large as 100,000 daltons.[17] Lactacin B is bactericidal against other lactobacilli, *L. bulgaricus, L. helveticus, L. leichmannii,* and *L. lactis*, and heat stable (100°C for 1 hour). Synthesis and immunity are apparently chromosomally located as *L. acidophilus* N2 lacks plasmid DNA.

L. acidophilus 88 produces lactacin F.[16] Lactacin B and lactacin F are chemically very similar, although in addition to the lactacin B indicators, Muriana and Klaenhammer[180] found lactacin F antagonistic against *Lactobacillus fermentum* and *Enterococcus faecalis*. Also, unlike lactacin B, lactacin F is plasmid encoded, associated with plasmids of approximately 6 and 40 kb. Genetic analysis of transconjugants indicated the involvement of transient plasmid determinants capable of chromosomal insertion, suggesting that the detectable plasmids were the result of a defective or incomplete mobilization event. Further study by Muriana and Klaenhammer[181,182] revealed lactacin F to be a micelle-like globular particle with a molecular weight of 180,000. Amino acid analysis estimated a peptide of 56 amino acids (~7,500 daltons); SDS-PAGE analysis approximated a size of 2,500 daltons. Purification of lactacin F is extremely difficult due to its highly hydrophobic nature. Lactacin F is extremely heat stable (121°C/15 minutes), which is indicative of a small hydrophobic peptide lacking tertiary structure. Indeed, lactacin F is so heat stable that it can easily be used as a selective agent in autoclavable bacteriological media to select transconjugants in matings employing lactacin F production and immunity as markers.[144] Lactacin F probably acts at the cell membrane.

Helveticin J differs from lactacins B and F. This bacteriocin is more closely related to caseicin 80[201] and acidophilucin A.[246] These bacteriocins are large and sensitive to inactivation by heat and have not been extensively studied. Helveticin J has a limited range of antagonistic activity, inhibiting some strains of *L. helveticus, L. bulgaricus,* and *L. lactis.* Joerger and Klaenhammer[133] concluded that like lactocin 27, the genes for production and immunity of helveticin J reside on the bacterial chromosome. At neutral pH, *L. helveticus* 481 produces helveticin J, which forms aggregates of over 300,000 daltons, but with purification, a protein of 37,000 daltons can be identified that retains functionality. Later work by Joerger and Klaenhammer[134] resulted in the cloning and sequencing of the structural gene for helveticin J.

Lactocin 27 is produced by *L. helveticus* LP27 and appears very similar to the bacteriocin produced by *L. fermenti* 466 and studied by De Klerk and Smit.[61] Lactocin 27 was originally described as a protein-lipopolysaccharide complex of over 200,000 daltons[247]; however, purification yielded a glycoprotein of 12,400 daltons.[248] Heat stable with no demonstratable loss of activity after 1 hour at 100°C, lactocin 27 is bacteriostatic against *L. helveticus* LS18 by inhibiting protein synthesis and by interfering with sodium and potassium transport.

West and Warner[255] found bacteriocin activity in *Lactobacillus plantarum* 1193, but the bacteriocin, plantaricin B, was not isolatable from liquid culture. Sensitive to proteolytic enzymes, antagonistic activity was reduced upon exposure to lipase and α-amylase. Inhibition occurred against selected lactic acid bacteria. *L. plantarum* C11 was found to produce plantaricin A.[52] It is similar to plantaricin B, in that the inhibitory effect is against other lactic acid bacteria, members of *Lactobacillus, Leuconostoc,* and *Pediococcus.* Plantaricin A is heat stable (100°C/30 minutes) and active over a pH range of 4.0 to 6.5. The purification and partial amino acid sequence of plantaricin S was recently reported.[132] Purification from the crude growth extract increases antibacterial activity of plantaricin S over 350,000-fold. Full activity of plantaricin S is dependent upon two distinct peptides, α and ß, that have apparent sizes of 26 and 24 amino acids, respectively. They have no apparent homology with each other.

Schillinger and Lucke[224] screened 221 strains of lactobacilli isolated from meat and found 23 of them to exhibit bacteriocin-like activity. From this work, *L. sake* Lb 706 was found to produce sakacin A, which shows considerable variability in its efficacy against target bacteria. Lactic acid bacteria and strains of *Listeria monocytogenes* and *Listeria innocua* are sensitive to the bactericidal action of sakacin A, but this sensitivity is highly dependent on the individual strain.[223] Antagonistic activity remains unaffected after a 20-minute exposure to 100°C, but all activity is eliminated after 20 minutes at 121°C. Sakacin A is a single polypeptide chain of 41 amino acids with a molecular weight of 4,309. Holck et al.[110] have sequenced sakacin A. These workers also found the structural gene for the bacteriocin residing on a 60-kb plasmid encoding for a primary translation product of 59 amino acids that is shortened to 41 amino acids. In the N-terminal end, sakacin A has a very close sequence homology with other nonlantibiotic bacteriocins from lactic acid bacteria.[222] Given its effectiveness against *L. monocytogenes, L. sake* Lb 706 and sakacin A have been recommended for use in fermented meats and meat products.

Lactocin S is produced by *L. sake* L45, a culture isolated from a meat fermentation by Mortvedt and Nes.[176] The bacteriocin is moderately heat stable and effective against selected Gram-positive bacteria, most of which are lactobacilli and other lactic acid bacteria. Lactocin S is composed of 33 amino acids.[177] Its structural gene is plasmid linked.[176] Two plasmids of approximately 50 and 34 kb are associated with production and immunity; however, there is variability between the plasmid genotype and the phenotypic characteristics, suggesting transpositional elements that cause instability and rearrangement of the genes. Recently, Mortvedt-Abildgaard et al.[178] reported on the activity and production of lactocin S. The pH of the culture was a key parameter in the inactivation of target cells. At pH >6.0, lactocin S-sensitive cells were unaffected by the presence of lactocin S. Production of lactocin S by *L. sake* L45 was eliminated by aerobic conditions, but anaerobically, production was increased 8- to 10-fold when pH was maintained at 5.0 in a fermentor compared with batch cultures. The addition of 1% ethanol to the growth medium resulted in a 2- to 4-fold increase in yield of lactocin S.

Carnobacterium and Leuconostoc

The genus *Carnobacterium* has been distinguished from *Lactobacillus*[45] and represents the atypically nonaciduric lactobacilli indigenous to refrigerated meats. Carnobacteria have been shown to produce bacteriocins with the potential for commercial development.[236] The bacteriocins produced by *Carnobacterium piscicola* LV17 are of interest because activity is detected early in the growth cycle.[2,3] Carnobacteriocin A is produced from a plasmid, pCP49 and carnobacteriocin B from pCP40 in *C. piscicola* LV17.[4] A third bacteriocin may be present in this strain.[236] The bacteriocins from *C. piscicola* LV17 are bactericidal against other lactic acid bacteria, enterococci, and *Listeria* spp. The bacteriocins are heat stable (100°C/30 minutes) and stable over a broad pH range (2–11).

Carnocin UI49 is a bacteriocin from a strain of *Carnobacterium* isolated from fish.[238] Amino acid analysis of the 4,635-dalton peptide suggests that carnocin UI49 belongs to the lantibiotic class of bacteriocins, making UI49 distinct from the bacteriocins produced by *C. piscicola* LV17 that do not contain lantibiotic-like amino acids.

Members of *Leuconostoc* are heterofermentative lactic acid bacteria commonly associated with fermented vegetables and cultured dairy products. Like carnobacteria, leuconostocs can predominate on chill-stored meats packaged with elevated levels of carbon dioxide.[227] Leucocin A is produced by *Leuconostoc gelidum* UAL 187.[101] The 3,930-dalton peptide is heat resistant (62°C/30 minutes) and inhibitory against lactic acid bacteria, *E. faecalis*, and *Listeria monocytogenes*. As with *C. piscicola* LV17, *L. gelidum* UAL 187 produces bacteriocin early in its growth cycle, but can additionally produce leucocin A during growth at 1°C. Mesenterocin 5 is produced by *Leuconostoc mesenteroides* UL5.[48] While mesenterocin 5 is a heat stable (100°C/30 minutes) 4,500-dalton peptide effective against *L. monocytogenes*, it is not produced early in growth.

Given the limitations of nisin and nisin-producing lactococci in meats, carnobacteriocins and bacteriocins from *Leuconostoc* have been suggested for development for storage extension and enhanced safety of chill-stored meats.[237] Although effective against most strains of *L. monocytogenes*, bacteriocins of *Carnobacterium* and *Leuconostoc* have a limited spectrum of activity. A bacteriocin with a broader antibacterial spectrum would be preferable.[236] Other disadvantages include a minimum pH for the synthesis and release of bacteriocins above the pH of the meat, and gas or dextran production by leuconostocs. An important advantage is that these bacteria are adapted to the intended products so growth and activity in the chilled meat products could occur. Certainly, the genetic engineering of carnobacteria and leuconostocs isolated from meat with more functional bacteriocin genes from other bacteria would have potential in this area. Allison et al.[6] recently introduced the lactacin F operon from *Lactobacillus johnsonii* VPI 11088 into *C. piscicola* LV17. The transformants of *C. piscicola* LV17 expressed lactacin F as well as the native carnobacteriocins, demonstrating that the machinery for carnobacteriocin production is capable of processing and exporting bacteriocins from both systems. Similarly, van Belkum and Stiles[251] introduced lactococcin A production (from *Lactococcus lactis* subspecies *cremoris*) into leucocin A-producing *Leuconostoc gelidum* UAL187 showing that lactococcin A could be exported by the leucocin A-secreting machinery.

Pediococci

Homofermentative pediococci serve as commercial meat starter cultures in the production of fermented sausages and have been found to produce bacteriocins with possible use as food preservatives. For example, pediocin AcH from *Pediococcus acidilactici* inhibits a much wider range of bacteria than bacteriocins from *Carnobacterium* and *Leuconostoc*. In addition to lactic acid bacteria and listeriae, pediocin AcH inhibits species and strains of *Bacillus* (both spores and vegetative cells), *Clostridium* (both spores and vegetative cells), *Staphylococcus*, *Propionibacterium*, and *Enterococcus*, as well as *Brochothrix thermosphacta*.[23] As shown with nisin, the bactericidal effect is dependent upon the ratio of the concentrations of the bacteriocin and the target organisms. Inhibition by pediocin AcH is highly variable within species of a genus and among strains of the same species. In each sensitive strain, some cells are resistant to pediocin AcH, ie, the percentage that is strain variable. Pediocin AcH acts on vegetative cells by membrane destabilization and upon spores by preventing outgrowth following germination.[205]

Both pediocin AcH and pediocin PA-1[89] are well-studied bacteriocins produced by different strains of *P. acidilactici*. Recently, they have been found to be the same peptide.[205] PA-1 is a simple protein of 44 amino acids (4,629 daltons) with two disulfide bonds.[105] Produced by *P. acidilactici* PAC 1.0, pediocin PA-1 is encoded by a 9,400-kb plasmid. Deletion analysis from cloning and expression in *E. coli*[161] revealed a 5,600-kb fragment necessary for pediocin PA-1 synthesis. Four open reading frames were found, of which two were characterized. The first encoding for the 62-amino acid precursor with amino acid residues 19 to 62 corresponding to the purified bacteriocin, and the fourth open reading frame specified a large protein of 724 amino acids required for pediocin PA-1 production in *E. coli* that probably corresponded to an ATP-dependent transport protein. Comparison of the amino acid sequence of pediocin PA-1 with leucocin A-UAL, lactacin F, and lactococcins A, B, and M found few sequence similarities except for the N-terminal leaders of amino acids 18 to 24 of the precursor proteins, particularly near the cleavage site. This site is uniquely conserved in the precursors of bacteriocins from lactic acid bacteria, suggesting the requirement for different processing enzymes in the maturation of small heat-sensitive bacteriocins produced by lactic acid bacteria.

A mixture of dried powder of growth extract from *P. acidilactici* PAC 1.0 and 10% nonfat dry milk was used[198] for evaluation as a preservative against the growth of *L. monocytogenes* LM01 in cottage cheese, half-and-half cream, and cheddar cheese soup for two weeks at 40°C. The mixture showed effective preservative effects over a pH range of 5.5 to 7.0 against 10^2 to 10^3 cfu g^{-1} of *L. monocytogenes* in the foods.

A new bacteriocin from pediocococci was recently described.[42] Produced by *P. acidilactici* L50 that was isolated from Spanish dry-fermented sausages, pediocin L50 shows a wide range of activity. It was effective against all Gram-positive bacteria tested; target organisms included members of *Bacillus, Clostridium, Staphylococcus, Listeria,* and *Propionibacterium*. Pediocin L50 is heat stable (100°C/40 minutes). It appears to contain modified amino acids but not lanthionine or methyllanthionine.

With pediococci used extensively as meat starter cultures, the efficacy of bacteriocin-producing pediococci has been evaluated in various model meat systems. Examples include Nielsen et al.[184] who examined radiation-sterilized fresh lean beef muscle inoculated with *L. monocytogenes* Scott A and treated with filter-sterilized extract from growth of a bacteriocin-producing commercial meat culture of *P. acidilactici.* The bacteriocin reduced the attachment of *L. monocytogenes* to the meat surface. The bacteriocin was functional on meat surfaces after a month of refrigerated storage. Berry et al.[20] challenged *L. monocytogenes* Scott A (inoculated at 10^8 cfu g^{-1}) with *Pediococcus* spp. strains JD1-23 and MP1-08 in summer sausage chubs and found a reduction of 2 $\log_{10}$ using a bacteriocin-producing culture and a reduction of 1 $\log_{10}$ with a bacteriocin-negative culture of *Pediococcus*. In a later study with frankfurters,[21] bacteriocin-producing *P. acidilactici* was deemed effective against *L. monocytogenes.* In the making of dry fermented sausages, bacteriocin-positive *P. acidilactici* PO2 eliminated a mixture of five strains of *L. monocytogenes* (inoculated at 10^5 cfu g^{-1}) when the pH at the end of the fermentation was <4.9.[75] To improve the effectiveness of pediocin AcH, Degnan and Luchansky[58] encapsulated the bacteriocin with phosphatidylcholine liposomes and found almost a 30% improvement in bactericidal effect versus *L. monocytogenes* in heated slurries of beef and beef tallow compared with slurries amended with free pediocin.

Propionibacterium and Bifidobacterium

The propionibacteria are not closely related taxonomically to the lactic acid bacteria used in foods; however, on a functional basis they display similar traits and thus have become valuable in food fermentations. Members of the genus *Propionibacterium* metabolize carbohydrates or lactic acid to propionic acid, acetic acid, and carbon dioxide.[15] They are aerotolerant anaerobes that function in the production of the nutty flavor and "eyes" of Swiss-type cheeses, serve as probiotics and silage adjuncts, in rum fermentations, in production of propionic acid from whey and manufacture of vitamin B_{12} as well as the food preservative, Microgard®.

Propionibacterium theonii P127 produces propionicin PLG-1.[155] Inactivated by exposure to 85°C for 30 minutes, the bacteriocin is stable at pH 3 to 9 with its greatest activity at pH 7.0. Unlike most other bacteriocins from GRAS acidulating bacteria, propionicin PLG-1 (9,300 daltons) inhibits a range of Gram-positive and Gram-negative bacteria, and some yeasts and molds. Microorganisms affected by PLG-1 include most propionibacteria, lactococci, lactobacilli, pediococci, pseudomonads, and some *Saccharomyces* strains, *Candida lipolytica,* and aspergilli. Glatz and coworkers have biochemically characterized and obtained the N-terminal sequence for PLG-1.[156,188]

Jenseniin G is produced by *Propionibacterium jensenii* P126.[91] It is heat stable (100°C/15 minutes) and active against dairy propionibacteria, lactococci, and lactobacilli. It has also been shown to be sporostatic against spores of *Clostridium botulinum.*[80] The lack of detectable plasmids in *P. jensenii* P126 suggests a chromosomal location for the gene(s) of jenseniin G.

Bifidobacteria are more closely related to propionibacteria than lactic acid bacteria. The major commercial use of *Bifidobacterium* is as a probiotic culture, as discussed earlier. Jao et al.[127] implied that the antibacterial effect of *Bifidobacterium bifidum* may be caused by the production of antibacterial substances in addition to acids. Anand et al.[8] found possible bacteriocin activity in *B. bifidum*, although cultures of Gram-positive and Gram-negative bacteria were inhibited by growth supernatants below pH 4.5 and fractionated metabolites singly or in combination did not display activity. Meghrous et al.[168] partially characterized a bacteriocin from an unspeciated *Bifidobacterium*. Active between pH 2.0 and 10.0 and heat resistant (121°C/15 minutes), lactic acid bacteria and clostridia were inhibited.

Streptococcus and Enterococcus

Bacteriocins STB 40 and STB 78 have been described from *Streptococcus thermophilus*, one of the two primary commercial yogurt-producing cultures.[231] These bacteriocins were heat stable, active in a pH range of 2.0 to 12.0, inactivated by proteolytic enzymes and by lipase, and estimated to be between 10,000 and 20,000 daltons. Inhibition was demonstrated against other strains of *S. thermophilus* and *Enterococcus*.

Bacteriocin St10 is only antagonistic toward other strains of *S. thermophilus*; it is produced by *S. thermophilus* St10.[41] It is heat stable (121°C/15 minutes), inactivated by α-amylase as well as proteolytic enzymes, and in its native state appears to have a molecular weight exceeding 100,000.

Thermophilin 13 is produced by *S. thermophilus* Sfi 13.[157] It is heat stable (100°C/1 hour), active in the pH range of 1.6 to 10.0, and is a peptide of about 4,000 daltons.

Enterocins, bacteriocins from *Enterococcus*, have been known for some time.[28,145] They are effective versus *L. monocytogenes*.[165] For example, enterocin E1A is bactericidal to strains of *L. monocytogenes* and other enterococci.[148] Enterocin 1146 (produced by *E. faecium* DPC1146) has shown a very strong and widespread effectiveness against *L. monocytogenes*, and against all *Listeria* tested[189,190]; however, enterocin 1146 can be inactivated by exposure to 60°C, especially at a pH approaching neutrality. Nonetheless, in an acidic food at the levels of *L. monocytogenes* naturally found in contaminated foods, there is potential for the addition of *E. faecium* DPC1146 to a fermented product as an adjunct to the normal starter culture(s).[107] A mutant of *Enterococcus faecalis* INIA 4, a bacteriocin-producing strain, has been adapted as a preservative agent against *Listeria innocua* in raw milk cheeses.[135]

12.4 CONCLUDING REMARKS

This chapter presented an overview of the antimicrobial compounds and conditions produced by microorganisms with importance or potential importance in the preservation of foods and beverages. In this area over the past decade, bacteriocins have received the greatest amount of interest and subsequent laboratory research. However, work continues to evolve and mature with the blending of old and new antimicrobial methods and components in modern-day food processing. Certainly, major advances in food preservation are likely to be seen with the application of the recombinant methods involving genetic engineering and protein engineering in microbiology, but these advances will be optimized for compatibility and synergy with other developments in food processing. Examples of these other somewhat nontraditional areas in food preservation include the use of nonmicrobially derived enzyme systems and components, irradiation, active packaging systems that possess antimicrobial activity and biosensors for enhanced product safety, minimally processed chilled food technology and cold pasteurization methods (eg, hydrostatic pressure processing and pulsed light treatment). Consumer demand continues for highly nutritious, fresh-like foods that possess a high margin of safety usually associated with heavily processed foods. Having this contradiction to ameliorate puts the onus on food scientists to be innovative in the preparation and delivery of safe, high quality food products. These requirements usually necessitate a specific blend of processing methods and aids for each category of product. Therefore, with the growth in the complexity and scope of food laws and product regulations, future developments for the use of microbially derived preservatives and protective cultures should be even more product specific than what is seen in current practice. Of course, the success of any new product or technology is determined in the marketplace. A new preservative must not only be effective for its intended purpose, but do so at an economically feasible cost. That has proven an imposing barrier for many new and innovative preservative methods.

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13 New and Emerging Physical Methods of Preservation

Grahame W. Gould

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13.1 INTRODUCTION

The main preservation techniques used currently act in one of three ways: (1) preventing access of microorganisms to foods, (2) inactivating them should they have gained access, or (3) preventing or slowing down their growth should they have gained access and not been inactivated. However, there has been increasing interest in modifying these approaches, or in developing new ones, with the objective of reducing the severity of the more extreme techniques. While many such developments have involved new uses of existing techniques in new combinations to inhibit the growth of microorganisms (hurdle technology) (see Chapter 14), the new developments have also included radically new physical procedures aimed at inactivating microorganisms in foods. While the mechanisms of action of some of the new procedures are well understood at the cell physiology or molecular levels, understanding of others remains empirical.[27]

The techniques mostly aim to offer full or partial alternatives to heat. They include the use of high hydrostatic pressure (ultrahigh pressure), high voltage electric discharges (electroporation), high-intensity laser or noncoherent light pulses, high-intensity magnetic field pulses, and the combination of mild heating with ultrasonication and slightly raised pressure (manothermosonication). Of these, the first (hydrostatic pressure) has captured a niche market that is continuing to grow. The other techniques remain to be exploited or to have their commercial viability proven.

13.2 HIGH HYDROSTATIC PRESSURE

13.2.1 Background

High hydrostatic pressures, above about 100 MPa (1 MegaPascal is equivalent to about 9.87 atmospheres; 10 bar; 0.1 kgf cm^{-2}; 145 lbf $inch^{-2}$), were originally shown to inactivate vegetative bacteria by Hite[48] nearly a hundred years ago and by workers[49] (reviewed by Heden[46]) at the beginning of the 20th century. Spores were found to be much more resistant, surviving pressures above 1,200 MPa.[6,72] These early studies also demonstrated that pressure treatment extended the keeping quality of some foods, including milk,[48] fruits, and vegetables.[49] However, technological constraints prevented commercial use of pressure for food decontamination until the 1980s.[77] At this time, engineering techniques had advanced to the extent that industrial processes were developed for the pressure pasteurization of a number of low pH foods, in which spores were not a problem, including jams, fruit juices, jellies, sauces, and fruit for yogurt,[113] dressings, and ingredients for ice creams.[68] At the same time, because pressure affects macromolecules (eg, denaturing proteins and modifying polysaccharide gels to bring about textural changes that differ from those caused by heat), the technology is presenting new opportunities for novel food texturization.[14,63,77] More recently, therefore, there has been a rapid expansion in research into the engineering technology, microbiology, materials science, and other food science aspects of pressure pasteurization.[68] This was followed by the introduction of new pressure-pasteurized foodstuffs (eg, chill-stored guacamole, dairy, fish, and meat products[89]), including some that have higher pH values than the jams and fruit juices that were marketed originally. The outlook for pressure-treated foods is widely expected to grow, probably through the capture of selected small-volume, high-value, niche markets rather than high-volume commodity markets.

13.2.2 Pressure Delivery

The use of high pressure for food processing involves the application of pressure isostatically, ie, the pressure is equal thoughout the material being processed. The procedures used are referred to as *cold isostatic processing* (CIP). These derive from forming techniques used in the metals, ceramics, carbon/graphite, and plastics industries, and they utilize pressures in the range from about 50 to about 600 MPa at near ambient temperatures. CIP contrasts with WIP (warm isostatic processing), which is another forming technique that operates at temperatures up to about 200°C and has not been found relevant to food processing. Hot isostatic processing (HIP) employs pressures up to about 400 MPa at temperatures as high as 2,200°C and is used in the metals and ceramics industries.

Pressure is applied conventionally, either directly by forcing liquid into the treatment chamber or indirectly by forcing a piston into the liquid-filled vessel containing the food. There is a transient temperature rise associated with pressurization, amounting to about 11C° at 400 MPa and 23C° at 800 MPa (for water[29]). The temperature rise dissipates at a rate dependent on the volume of the treatment chamber, its construction materials, and the conductivity of the surroundings, etc. High pressure processing of foods in the first commercial systems to be developed operates as a batch process with treatment times commonly between about 0.5 and 5 minutes. The economics of the process demands fast cycling times and the highest pressures possible, currently up to about 600 MPa, commensurate with the avoidance of metal fatigue, too-frequent seal failure, etc.[77] These requirements pose substantial engineering challenges that have been met with a number of innovative approaches to vessel design, and that require regular replacement schedules for sensitive parts, for laboratory scale equipment,[30] and for commercial use.[21,77,85]

The volumes of commercial treatment vessels are currently between about 50 and about 1,000 L.[5] Larger capacity vessels are mostly limited to operating pressures lower than those capable of inactivating microorganisms in foods. True continuous processes are not yet used commercially, though cost-effective semicontinuous systems that can be used with pumpable liquid products have been developed.[5,81]

In contrast to the situation with thermal processing, the applied pressure is transmitted instantaneously and uniformly throughout the the treatment vessel and product being treated. No pressure gradients are established during the process. Product to be treated is normally enclosed in sealed packages, which are sufficiently flexible to withstand the compression that occurs during pressurization. The pressure medium surrounding the packs (usually water mixed with a small amount of oil for lubrication and additives to prevent corrosion of the steel vessels, pistons, and valves) and the packs and pack contents, are compressed to about 80% to 90% of their original volumes during pressurization in the 400 to 800 MPa region but, of course, return to their original volumes when the pressure is released.

13.2.3 Mechanism of Action

The effects of high pressure derive from the Le Chatelier principle.[40] Pressure favors any physical or chemical reaction in which there is a net volume decrease and suppresses any reaction in which there is a net volume increase. In kinetic terms, reactions with a negative activation volume will be accelerated while those with a positive activation volume will be retarded. In complex systems, such as a foodstuff or a microbial cell, in which many reactions take place, the overall effects will therefore depend on these terms rather than on the more familiar free energy or activation free energy that form the basis for the major effects of heat. With reference to biological systems, the most important volume-decrease reactions include the denaturation of proteins, gelation, hydrophobic interactions, phase changes in lipids (and therefore in cell membrane structure), and increases in the ionization of dissociable molecules resulting from electrostriction.[47] Small molecules are therefore generally less affected than macromolecules, so that low molecular weight (MW) flavor and odor compounds in foods tend to survive the pressure used to process them relatively unchanged, with consequent retention of high organoleptic quality of some types of foods (eg, jams).[55] On the other hand, the high MW components of some foods (eg, proteins in meat, fish, and eggs) change greatly on pressurization, and the changes that occur, particularly in texture, may or may not be desirable.[63]

It is clear therefore from the known action of pressure on molecules and chemical reactions that a structure as complex as a microorganism will contain a number of pressure-sensitive components and targets for lethality, eg, key enzymes, genetic material, and molecular assemblies such as ribosomes and membranes.[59] It is therefore not surprising that pressure induces a variety of changes in vegetative microbial cells,[54] eg, in the morphology of yeast mitochondria and cell walls and other subcellular structures and in cell wall–cell membrane interactions in bacteria.[67,70] Pressure caused changes in fatty acid composition and in the protease and lipase activities of *Yarrowia lipolytica*.[71] Many cellular enzymes will, of course, be affected by pressure. The effect of pressure on 13 metabolic enzymes produced by strains of *Listeria monocytogenes* varied greatly,[116] but did not correlate with barotolerance of the strains.[117] An important specific example includes membrane-bound Na/K ATPase.[16] Loss of this enzyme may play a part in the inactivation of vegetative bacteria by pressure through interference with pH homeostasis and consequent acidification of the cytoplasm.[119] However, the multiple effects of pressure make the existence of a single key site for pressure inactivation unlikely.

The situation regarding bacterial spores is different from that with vegetative cells. Ungerminated spores are very pressure tolerant,[106] but there is a region of pressure-temperature combinations where pressure directly causes spores to germinate.[16,37] This occurs even in the absence of any nutrients or chemical "germinants." Having been germinated by pressure, the germinated spores may then survive if the pressure and/or temperature are low enough or be inactivated if they are high enough. The combination of possibilities leads to a peculiar pattern of spore response to pressure in which relatively low pressures may be more sporicidal than higher ones under some circumstances (see below).

Regarding mechanisms of spore tolerance to high pressure, it was very reasonably suggested that this most likely resulted from protection of spore proteins by the low state of hydration of the cytoplasm in the spore protoplast.[123] The mechanism of action of pressure-induced germination is less clear. Its kinetics suggested a physiological basis, such as a shift in the equilibrium of a Donnan phase somewhere within the spore.[17] It has been proposed that the extreme dormancy of spores may result in part from their low water content protoplasts being in a glassy state[36] and that pressure, like temperature, would affect its stability. A glassy state was proposed, with some supporting kinetic evidence, to explain in part the heat resistance of spores.[110] Limited studies indicate that viruses have high tolerance to pressure.[14]

13.2.4 Effects on Microorganisms

Vegetative Cells

Kinetics. While the earliest studies of the effects of pressure on microorganisms showed that vegetative cells in milk, meat, fruits, and vegetables could be inactivated at about 300 MPa and above, there were few data on the kinetics of inactivation and the levels of kill achievable. More detailed studies have been made recently and, although some examples of exponential kinetics have been reported for the inactivation of vegetative cells held at constant pressure (eg, *Escherichia coli*),[11,74] the majority of studies have found "tails" on survivor curves (ie, a decreasing death rate with time of treatment) (Figure 13-1).[26,79,93] It has been suggested that a reason for these apparent discrepancies is that at higher temperatures (eg, 40°C and above for *E. coli* at 250 MPa) inactivation is near first order whereas at lower temperatures (eg, 30°C for *E. coli*) it is nearer to second order. It was proposed that temperature-dependent changes in lipid composition may account for these differences and, indeed, phase changes in membrane lipids have been shown to occur at around 30°C in *E. coli*.[28]

Temperature, Water Activity, and pH. More generally, over the temperature range from +20°C to –20°C high pressure is more effective in inactivating vegetative microorganisms at the lower than the higher temperatures (eg, for *Lactobacillus casei* and *E. coli*[120] and for *Staphylococcus aureus, Salmonella bareilly*, and *Vibrio parahaemolyticus* in phosphate buffers[122]). Likewise, *Citrobacter freundii* in minced beef was more pressure sensitive at 4°C than at 20°C (a 10^6-fold reduction was caused by pressurization at 280 MPa for 20 minutes at 20°C, but 230 MPa caused

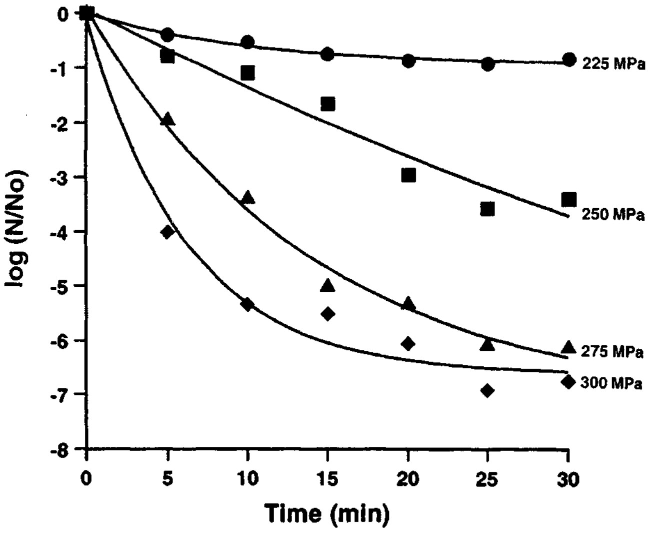

Figure 13-1 Nonlinearity of Pressure Survivor Curves. *Yersinia enterocolitica* in pH 7 phosphate-buffered saline at 20°C. *Source:* Reprinted with permission from M.F. Patterson et al., Effects of High Pressure on Vegetative Pathogens, in *High Pressure Processing of Foods,* D.A. Ledward, D.E. Johnston, R.G. Earnshaw, and A.P.M. Hasting, eds., pp. 47–63, © 1995, Nottingham University Press.

the same reduction at 4°C[12]). It has been suggested that, in addition to any direct effects (eg, on the fluidity of membrane lipids etc), low temperature may prevent metabolic adaptation to pressure and so minimize any rise in tolerance occurring during the period of holding under pressure.[26]

While salt solutions have been reported to reduce the pressure resistance of some vegetative microorganisms in buffers,[23] effects in foods are more complex. Generally, it has been found that substantial reductions in water activity (a_w), achieved by the addition of a range of solutes (including sucrose, glucose, and fructose, of relevance in the pressure pasteurization of jams, as well as sodium chloride) led to increases in pressure tolerance, eg, of *Rhodotorula rubra*.[88] While pH over the range 3 to 8 did not influence the pressure inactivation of this organism, raising the treatment temperature to 45°C allowed a satisfactory pasteurization process to be achieved at 400 MPa. The low pH– and low a_w–tolerant yeast, *Zygosaccharomyces bailii,* which frequently spoils products such as mayonnaises, pickles, and fruit concentrates, was reduced more than 10^6-fold by treatment at 345 MPa at 21°C for 5 minutes when the a_w was higher than 0.98. However, at lower a_w, achieved by the addition of sucrose, the level of inactivation was much less, eg, about 10^2-fold at a_w 0.92.[91] The baroprotective effect of reduced a_w was described by a linear relationship between $(1 - a_w)$ and the log of the surviving fraction.

Influence of Food and Strain. There are a number of examples where the pressure tolerance of a particular microorganism varies greatly in different media or foods, for reasons that remain unclear (Figure 13-2). For example, *Salmonella typhimurium* was inactivated about 10^6-fold in pork meat by a 10 minute pressure treatment at 300 MPa whereas only a 10^2-fold reduction was achieved in a chicken baby food at the slightly higher pressure of 350 MPa.[79] *Listeria monocytogenes* was more resistant to pressurization in UHT milk than in phosphate buffered saline.[121]

Strain-to-strain variation in pressure sensitivity seems to be greater than the strain-to-strain variation in sensitivity to other inactivation techniques, such as heat. For example, *L. monocytogenes* 4b serotypes NCTC 11994, Scott A, and an isolate from chicken were inactivated by just less than 1 log, just over 2 logs, and about 5 logs, respectively, by pressurization at 375 MPa for 10 minutes at 20°C in 10 mM phosphate-buffered saline (Figure 13-3).[94]

Otherwise, exponential phase cells are more pressure sensitive than stationary phase ones.[23,59] Gener-

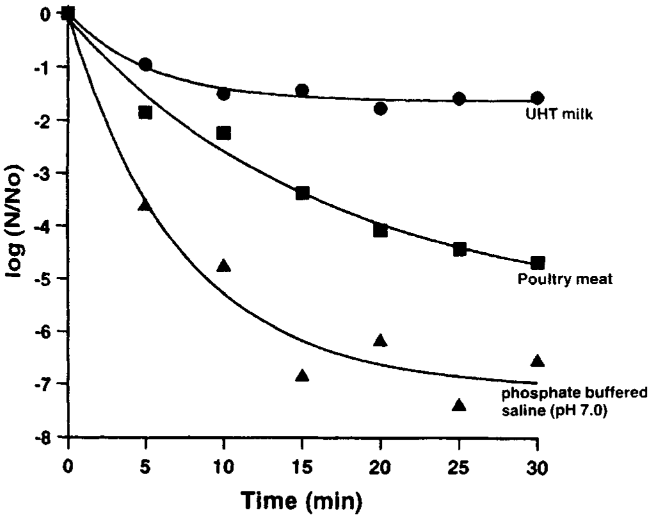

Figure 13-2 Influence of Food and Environment on Pressure Tolerance of Microorganisms. *Escherichia coli* O157:H7, pressurized at 700 MPa at 20°C. *Source:* Reprinted with permission from M.F. Patterson et al., Effects of High Pressure on Vegetative Pathogens, in *High Pressure Processing of Foods,* D.A. Ledward, D.E. Johnston, R.G. Earnshaw, and A.P.M. Hasting, eds., pp. 47–63, © 1995, Nottingham University Press.

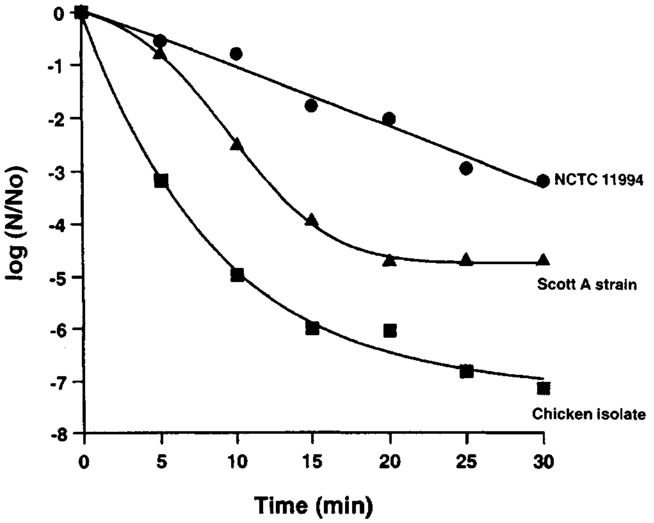

Figure 13-3 Strain Differences in Pressure Tolerance. *Listeria monocytogenes* pressurized in pH 7 phosphate-buffered saline at 375 MPa at 20°C. *Source:* Reprinted with permission from M.F. Patterson et al., Effects of High Pressure on Vegetative Pathogens, in *High Pressure Processing of Foods*, D.A. Ledward, D.E. Johnston, R.G. Earnshaw, and A.P.M. Hasting, eds., pp. 47–63, © 1995, Nottingham University Press.

ally Gram-positive microorganisms are more pressure tolerant than Gram-negative ones.[115] For example, *Staphylococcus carnosus* was inactivated by less than 1 log by exposure to 400 MPa for 1 hour,[27] and evidence of recovery was seen in a rising count during continued pressurization. However, important exceptions to the generally observed lower resistance of Gram-negative bacteria have been observed (eg, *E. coli* O157:H7, see below).

Pathogens. Reported pressure sensitivities of foodborne vegetative pathogens in a variety of buffers, media, and foods were reported by Patterson et al.[93,94] Pressurization times were mostly within the range from 10 to 30 minutes and at temperatures near to 20°C. The most sensitive pathogen was *Vibrio parahaemolyticus*, with a 10^6-fold reduction at pressures as low as 170 MPa.[121] *Yersinia enterocolitica* and *Campylobacter jejuni* were similarly inactivated at 300 MPa[115] and *S. bareilly* (>10^8-fold) at 200 MPa.[122] Exposure to 300 MPa inactivated *Salmonella typhimurium* 10^6-fold as enumerated on selective agar[115] but less than 10^2-fold when enumerated on nonselective medium.[79] A similar pattern was found for *E. coli*.[59] *Salmonella senftenberg* 775W and *Salmonella enteritidis* were slightly more resistant, with 10^4- to 10^6-fold reductions at 350 to 500 MPa.[94] *L. monocytogenes* Scott A was inactivated 10^6-fold or more in a variety of media and foods at pressures ranging from 340 to 450 MPa. *S. aureus* was inactivated more than 10^8-fold in phosphate buffer at 400 MPa but only 10^4-fold in poultry meat and 10^2-fold in UHT milk at 600 MPa.[94] The organism most out-of-line with the general sequence of tolerance from Gram-negative to Gram-positive organisms was *E. coli* O157:H7 for which the very high pressure of 700 MPa was necessary to achieve a 10^6-fold reduction in phosphate-buffered saline and a 10^5-fold reduction in poultry meat (Figure 13-2). In UHT milk, even exposure at 800 MPa failed to affect more than a 10^2-fold reduction.[94]

Injury and the Effect of Stresses. The lower recovery of pressurized microorganisms on selective versus nonselective media referred to above illustrates that pressure can cause nonlethal injury that is only expressed under conditions of stress.[64] Such food-relevant stresses may include mild heating and reduced pH. A potentially useful stress has been shown to be the presence of a bacteriocin such as nisin or pediocin. These amplified the inactivation by pressure of *L. monocytogenes* Scott A, *E. coli* O157:H7, and *S. typhimurium*.[64] High pressure transiently sensitized *E. coli* to nisin and lysozyme.[44] Chitosans increased the effectiveness of high pressure against *E. coli, S. aureus*, and *Saccharomyces cerevisiae*.[92] Although normally lysozyme resistant, *E. coli* was more pressure sensitive in its presence than in its absence.[95] On the other hand, it has been shown that imposition of stress on microorganisms prior to pressure treatment may increase their subsequent pressure tolerance. For example, cycles of pressure treatment of *E. coli*, followed by growth of survivors, led to a population exhibiting increased pressure tolerance.[43] Sublethal heating (heat shock), as expected, raised the resistance of *S. cerevisiae* to subsequent heating at a higher temperature, but also raised its resistance to hydrostatic pressure.[60] Mutants of *S. cerevisiae* lacking the ability to accumulate trehalose and/or to synthesize certain heat-shock proteins, had lost the heat-shock response, as expected, but also became sensitive to pressure.[61] Mutants of *E. coli* with increased pressure resistance were isolated from pressure-treated cultures. The mutants were inactivated by no more than 2 logs at pressures up to 600 MPa (15 minutes at ambient temperature) whereas the wild type was inactivated to a greater extent at 420 MPa under the same conditions.[43]

Altogether these various influences of environment and to a lesser extent, temperature, mean that it is currently much more difficult to predict the exact effects on microbial survival of a particular pressure treatment in a particular food than, for example, a particular heat treatment. Furthermore, the apparently large strain-to-strain differences in pressure tolerance make predictions for specific microorganisms even more difficult. Extensive *inoculated pack* studies remain necessary, particularly for low acid–high water activity foods in which the presence of pathogens is possible.

Spores

The extreme pressure tolerance of bacterial endospores was first demonstrated over half a century ago.[72] Pressures up to 1,200 MPa failed to inactivate spores of a number of species.[6,72,123] However, it was discovered later that, under certain conditions, inactivation of spores occurred more rapidly and completely at lower than at higher pressures.[17,106] These observations were difficult to reconcile with the expected effect of a physical treatment on a biological system: an increase in the intensity of a treatment would normally be expected to result in an increase in the degree of inactivation, comparably with heat, ultraviolet and ionizing radiations, electric shock, ballistic disintegration, etc.[102]

An explanation for the peculiar effect of pressure was found when it was observed that inactivation of spores occurred in two stages.[17,37] First, pressure caused the spores to germinate. Second, pressure inactivated the germinated forms. Spores of some species could be caused to germinate by pressures as low as a few tens of MPa, too low to inactivate the germinated forms. Under these conditions, the effect of pressure was therefore nonlethal, but brought about the normal range of sensitivities that are associated with spore germination (eg, to heat and to irradiation). This led to exploration of the combined use of pressure in combination with raised temperature[106] or with low dose ionizing radiation[127] to achieve a higher level of spore inactivation.

The fact that there was an optimum pressure for germination meant that as the pressure was raised above the range that caused the most rapid germination, the germinative effect was lost. However, the germinative effect of pressure was found to be very temperature dependent, so that the effect of combining pressure and temperature was complex, with an overall pattern like that shown in Figure 13-4A. If, following the release of pressure, the treated spores were subjected to a mild pasteurization heat treatment (eg, 70°C for 30 minutes), then a further fall in count occurred as a result of heat inactivation of the spores that had been germinated but not inactivated by the particular pressure-heat combination treatment (Figure 13-4B). Recent studies have confirmed the efficacy of pressure-temperature combinations in synergistically inactivating a wide range of spore types.[45,65,69,75,83,114]

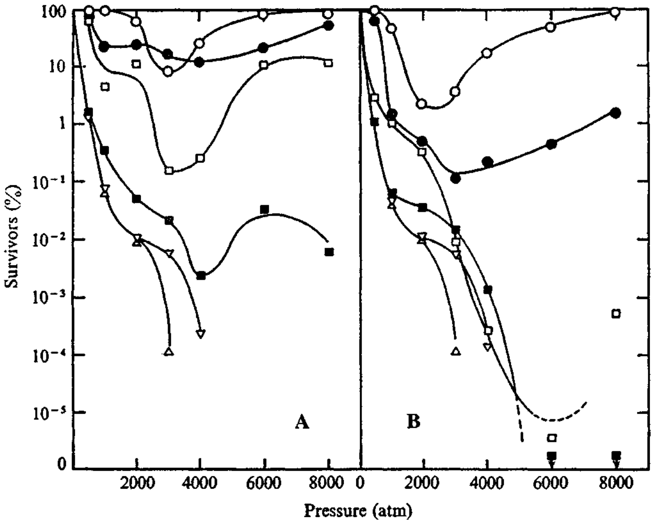

Figure 13-4 Inactivation of Spores of *Bacillus coagulans* at Different Combined Pressures and Temperatures. Spores in sodium phosphate buffer (100 mM, pH 8) were pressurized for 30 minutes at 25° (○), 35° (●), 45° (□), 55° (■), 65° (△) and 75°C (▽). (**A**) Direct count after depressurization. (**B**) Counts after a post-pressurization heat treatment at 70°C for 30 minutes. *Source:* Reprinted with permission from A.J.H. Sale, G.W. Gould, and W.A. Hamilton, Inactivation of Bacterial Spores by Hydrostatic Pressure, *Journal of General Microbiology,* Vol. 60, pp. 323–334, © 1970, Society for General Microbiology.

Pressure cycling has been suggested as one approach to increase the inactivation of spores. Six 5-minute cycles of oscillating pressure at 600 MPa and 70°C reduced *Bacillus stearothermophilus* spore numbers by 6 logs.[45] However, others have found that the effect of pressure on spores results mainly from integrated time at pressure rather than on the act of raising or lowering pressure.[106,114]

The kinetics of germination/inactivation of spores of *Bacillus pumilus* by pressure was near exponential,[18] but spores of *Bacillus coagulans, Bacillus subtilis,* and *Clostridium sporogenes* held at constant pressure and temperature showed nonlog/linear inactivation kinetics, most often with concave-upward curves or long tails.[106]

Although the mechanism by which pressure causes germination is not yet known, it appears to be physiological in being most effective at near-neutral pH values and inhibited by extremes of pH when pressures are low, though *B. coagulans* spores were shown to be more pressure sensitive at pH 4 than at pH 7 at high pressure (400 MPa).[101] Pressure germination is inhibited at low water activities, most effectively if the low a_w is achieved by high concentrations of ionic solutes, such as NaCl, rather than nonionic ones, such as sucrose and glucose.[106] The germinative effect of pressure is increased by low levels of the physiological germinants such as L-alanine, inosine, and glucose, which are specific triggers for the germination of different types of spores. Pressure germination is inhibited by inhibitors that prevent such physiological germination.[37] However, as the pressure and temperature are raised in combination treatments, the pressure effects become dominant and added germinants and inhibitors exert less and less influence. The relationship of germination to pressure tolerance was further illustrated by the isolation of pressure-resistant mutants of *B. subtilis*. These were found to be not only pressure tolerant, but also defective in one of the known physiological germination systems (the *alanine pathway*) at ambient pressure.[129]

While Figure 13-4, which summarizes data obtained with *B. coagulans*, represents the pattern of response shown by all types of spores that have been studied, the exact levels of inactivation depend also on food-specific parameters such as pH, a_w, and nutritional status. Furthermore, the sensitivity of spores of different species varies *greatly*. For example, spores of *B. cereus* were much more sensitive than those illustrated in Figure 13-4, while spores of *Clostridium botulinum*, *B. subtilis*, and *B. stearothermophilus* were much more resistant,[67] so that generalizations must be treated with caution.

The extreme resistance of some types of spores has so far prevented the use of pressure to sterilize foods.[53] All current commercial applications are *pressure pasteurization* processes. The use of pressure to achieve sterilization must await either the development of economically viable presses that operate at higher pressure and temperature combinations, or the development of some alternative effective combination techniques.[14] For example, it has been shown that, as with vegetative cells, the presence of bacteriocins such as nisin can enhance the effectiveness of pressure against spores, eg, of *B. coagulans*, particularly if coupled with reduced pH (from 7 to 4) and raised temperature.[101]

The antimicrobial effectiveness of CO_2 is enhanced at moderate pressures. Microbial numbers on fresh herbs were reduced over 10^5-fold by treatment at 55 MPa under CO_2,[39] but most likely due to cell disruption caused by the intracellular release of dissolved gas on the release of pressure rather than the direct effect of pressure itself.[73]

13.3 HIGH VOLTAGE ELECTRIC DISCHARGE

13.3.1 Background

Pasteurization of foodstuffs by electric fields was first described by Getchell in 1935.[34] It was shown that passage of an alternating current through milk inactivated microorganisms in it. This probably occurred mainly through heating, but it was claimed that additional nonthermal inactivation occurred as well. While the application of electric fields to heat foods has since become well established,[90] eg, through electrical resistance or "ohmic" heating[32] and microwave heating,[82] the use of electric pulses to effect nonthermal inactivation of microorganisms in foods was explored and exploited more recently.[13] At the same time, use of the technique at lower, nonlethal voltages has become established as the basis for *electroporation*, by which genetic material may be exchanged between protoplasted cells of microorganisms, plants, and animals.[84]

A method was patented in 1960 for inactivating microorganisms with an electric field.[22] Later studies demonstrated the inactivation of bacteria and yeasts and the lysis of protoplasts and erythrocytes.[107,108] Still later studies concentrated on varying the electrical parameters[56,57,58] and the effects of the medium/environment.[62,80] These studies led to the development of prototype and industrial scale devices in the late 1980s and early 1990s (see below).

13.3.2 Delivery of High Voltage Discharges

Electric fields may be delivered as oscillating, bipolar, exponentially decaying, or square wave pulses. Oscillatory pulses have been found to be the least effective, while the energy efficiency of square wave pulses results in them being more lethal than exponentially decaying ones. Bipolar pulses (ie, with a reversal in the polarity of the field) were more lethal than monopolar pulses because, it was presumed, rapid reversal in the direction of movement of charged molecules caused greater damage to cell membranes. Additional advantages of bipolar pulses are that they generate less electrolysis in foods and they are energy efficient.[99]

The strength of the electric field which, along with the size of the microbial cell, determines the potential difference that develops across the cell membrane, is more important than the duration of the pulse in determining the extent of inactivation. Consequently, it is generally most economic to raise the field strength as high as possible while reducing the duration of the pulses, without increasing the pulse energy.[38] On the other hand, from the practical point of view, the use of very high field strengths demands more complex and expensive engineering.[130]

As a result of these competing requirements, the more modern pulse-field devices employ field strengths from about 20 up to about 70 kV cm^{-1}, with pulse durations of between 1 and about 5 microseconds. Pulse repetition rates and overall treatment times are limited in order to minimize temperature rise in the food being treated. Repetition rates are commonly between 1 and up to 30 seconds or so at the higher voltage gradients. As a result of these requirements, the engineering objectives have been to design treatment chambers to hold foods in such a way that they receive near-uniform treatment, with pulses that are of short duration but have high-intensity field strengths and cause minimal electrolysis. The chambers may operate batchwise or continuously. The earliest versions operated batchwise. They were not fully enclosed and so were limited to a field strength of about 25 kV cm^{-1} because this was the approximate breakdown voltage of air.[25,107] Enclosure and design improvements led to batchwise devices that could deliver 30 to 40 kV cm^{-1}.[38,130] These were useful for laboratory studies to determine the optimum parameters for the inactivation of microorganisms. However, continuous operation is necessary for cost-effective industrial systems able to treat liquids or liquids containing particulates. A number of such systems, mostly designed around coaxial cylindrical electrodes, were therefore developed.[7,10,25,51,100,111,113,118]

13.3.3 Mechanism of Action

While the effects of high voltage fields on microorganisms are not understood at the molecular level, the gross effects, and the mechanisms that cause them, are well established. They result from permeabilization of the cell membrane.[41] Microbial cells act as insulators due to the low conductivities of their membranes so that, at low field strengths, they resist the effects of electric pulses. The field is effectively excluded from the microbial cell. As the field strength is raised, however, it becomes sufficient to overcome the resistance of the membrane in such a way that the voltage gradient across the whole cell is concentrated across the membrane at the two ends of the cell that are perpendicular with respect to the field gradient (Figure 13-5). It has been established that breakdown results when the voltage drop across the membrane reaches about 1 volt.[15,35] Although this may seem small, the fact that the membrane is only a few tens of Ångstroms across, results in the development of an enormous voltage gradient across it. It will be clear from Figure 13-5 that large cells (eg, yeasts) will therefore be expected to be killed at lower field strengths than small cells (eg, micrococci) and, indeed, this has been found to be so.[41]

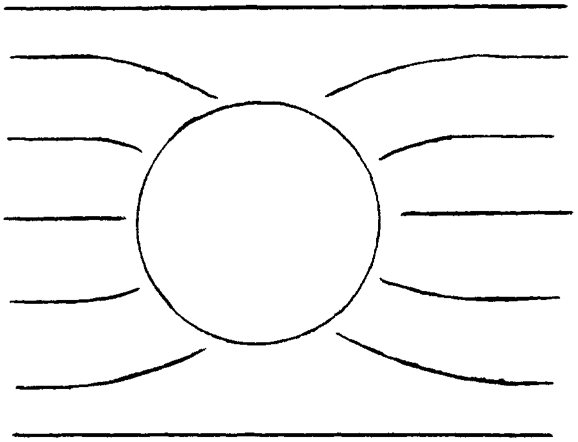

Figure 13-5 Diagrammatic Representation of the Influence of the Cell Membrane on the Equi-Potential Lines during High Voltage Discharge Treatment. The arrow indicates the direction of the applied field. *Source:* Reprinted with permission from A.J.H. Sale and W.A. Hamilton, Effects of High Electric Fields on Microorganisms II: Lysis of Erythrocytes and Protoplasts, *Biochimica et Biophysica Acta,* Vol. 163, pp. 37–45, © 1968, Elsevier Applied Science.

The result of the membrane damage is to make the cell temporarily leaky. It may survive if the damage is not extreme; this is the basis of electroporation. At higher voltages massive leakage of cell contents occurs and the cell dies.[124]

13.3.4 Effects on Microorganisms

Vegetative cells of bacteria, yeasts, and molds are inactivated by sufficiently high electric field pulses as a direct result of membrane permeabilization. Pulsed-field inactivation has been reported for *E. coli, S. typhimurium, S. dublin, Streptococcus thermophilus, Lactobacillus brevis, Pseudomonas fragi, Klebsiella pneumoniae, S. aureus, L. monocytogenes, S. cerevisiae,* and *Candida albicans.*[5] In contrast to vegetative organisms, bacterial spores[107] and yeast ascospores[78] were resistant, even at voltage gradients above 30 kV cm^{-1}, though recently the first reports of spore inactivation have been made.[76] The effect of increasing voltage gradients is illustrated in Figure 13-6 for a number of bacteria and yeasts. The data show the large differences in the resistance of different microorganisms and illustrate the general observation that large-celled species are more sensitive than small-celled ones.

With respect to the inactivation of microorganisms in foods, a number of controllable intrinsic and extrinsic factors influence the effectiveness of electrical treatments. Inactivation increased greatly with increase in temperature, eg, for *E. coli* between 3° and 40°C (Table 13-1).[100] Similar data were reported for *Lactobacillus brevis*[62] over the range 24° to 60°C, but with the probability of some thermal inactivation at the higher end of the temperature range. Low ionic strength favors inactivation by pulsed electric fields such that a reduction in the KCl concentration in simulated skim milk from 0.17 to 0.03 M resulted in a fall in the surviving fraction of *E. coli* following a 55 kV cm^{-1}, 20 pulse treatment, from about 0.3 to 0.002.[125] Reduction in pH value increased inactivation, eg, it was approximately doubled for *E. coli* when the pH of skim milk was reduced from 6.8 to 5.7.[125] Log-phase cells are more sensitive than stationary-phase cells.[125] Application of electric treatments to liquid foods has indicated that useful *cold pasteurization* inactivation of yeasts and vegetative microorganisms can be achieved. For example, treatment of apple juice, at temperatures below 30°C, with less than 10 pulses in a continuous treatment chamber, caused >6 log reduction of *Saccharomyces cerevisiae* at voltage gradients of 35 kV cm^{-1}; 22 kV cm^{-1} delivered just over 2 log inactivation.[98]

Table 13-1 Effect of Temperature on the Inactivation of *Escherichia coli* by High Voltage Electric Discharge Treatment (50 kV cm^{-1}, 2 μsec pulses) in Simulated Skim Milk

	Surviving Fraction after Total Treatment Times (microsec) of		
Temperature (°C)	*10*	*20*	*100*
3	1.0	0.9	0.3
7	0.9	0.6	0.1
15	0.7	0.3	0.03
22	0.5	0.16	0.007
30	0.3	0.08	0.001
40	0.1	0.008	<0.001

Source: Reprinted with permission from B. Qin et al., Nonthermal Pasteurization of Liquid Foods Using High-Intensity Pulsed Electric Fields, *Critical Reviews in Food Science and Nutrition*, Vol. 36, pp. 603–607, © 1996, CRC Press, Inc.

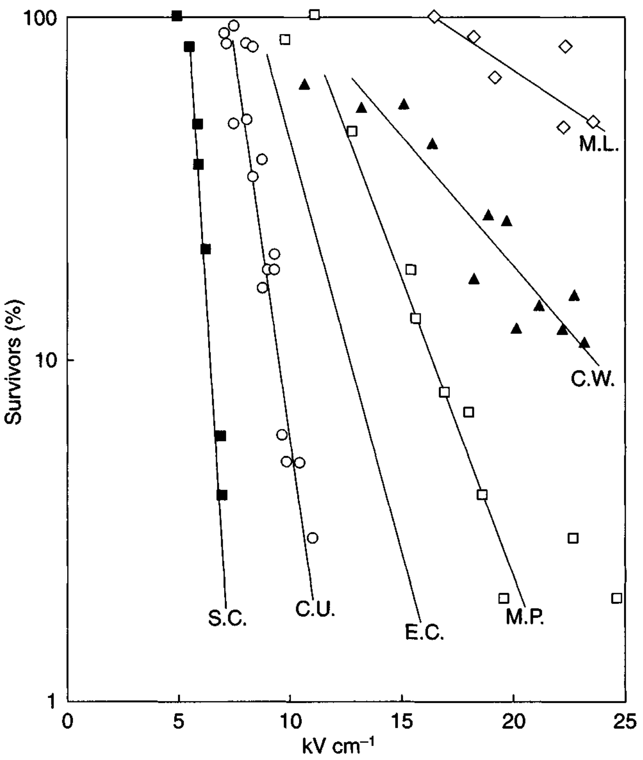

Figure 13-6 Inactivation of Different Vegetative Microorganisms by High Voltage Electric Field Pulses at Different Voltage Gradients. 10 pulses of 20 microseconds. Organisms: SC, *Saccharomyces cerevisiae*; CU, *Candida utilis*; EC, *Escherichia coli*; MP, motile pseudomonad; CW, *Clostridium perfringens*; ML, *Micrococcus lysodiekticus*. *Source:* Reprinted with permission from A.J.H. Sale and W.A. Hamilton, Effects of High Electric Fields on Microorganisms II: Lysis of Erythrocytes and Protoplasts, *Biochimica et Biophysica Acta*, Vol. 163, pp. 37–45, © 1968, Elsevier Applied Science.

There is a problem in the commercial use of high voltage pulse technology that results from the kinetics of inactivation. Studies of inactivation rates under a variety of conditions have generally indicated kinetics that, on a log survivor versus treatment time, or versus number of pulses basis, show long "tails." Near straight lines are seen when log survivors are plotted against the log of the treatment time, or against the log of the number of pulses (Figure 13-7).[131] This means that whereas a small number of pulses may satisfactorily treat a food if a small number of log reductions in the target organism is required, the number of pulses needed increases greatly if high log reductions are required. For example, from Figure 13-7, *E. coli* would be about 10^3-

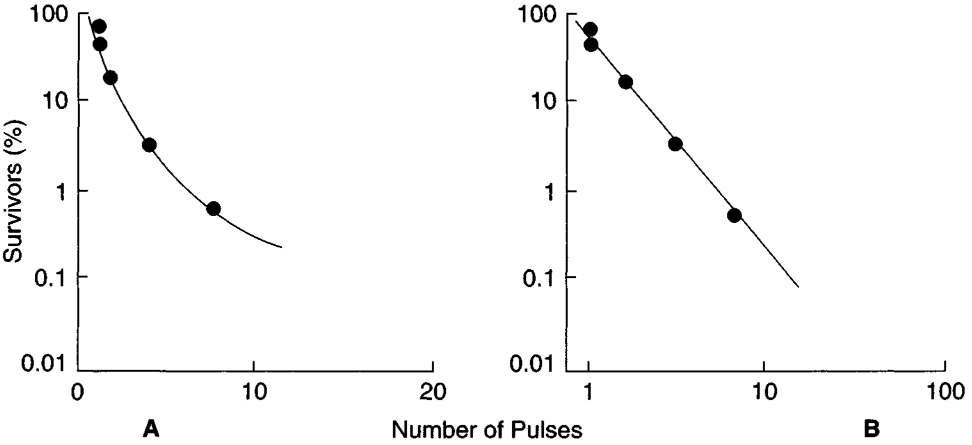

Figure 13-7 Inactivation Kinetics of *Escherichia coli* in Simulated Milk Ultra-Filtrate at 20°C by 35 kV cm^{-1}, 2 microsec Pulses. (**A**) Log survivor:linear dose plot. (**B**) Log survivor:log dose plot. *Source:* Adapted with permission from Q. Zhang et al., Inactivation of *E. coli* for Food Pasteurization by High-Strength Pulsed Electric Fields, *Journal of Food Processing and Preservation,* Vol. 19, pp. 103–118, © 1995, Food and Nutrition Press, Inc.

fold inactivated by 10 pulses under the conditions indicated but (extrapolating) more than 100 pulses would be required to achieve a 10^6-fold inactivation, and more than 1,000 to achieve a 10^9-fold kill. Nevertheless, at higher field strengths (eg, 70 kV cm^{-1}) 10^9-fold kills can be achieved with 100 pulses (*E. coli* at 20°C). This is judged to be an economically feasible basis for commercial cold pasteurization of pumpable liquid foods, with the advantages of minimal changes in flavor, color, and texture.[131,132]

While most research into the effects of electric pulses on microoganisms has involved single treatments, studies of combination processes have shown potentially useful synergies. For example, electroporated cells of *L. monocytogenes, E. coli,* and *S. typhimurium* become much more sensitive than untreated cells to nisin and pediocin.[64]

13.4 HIGH-INTENSITY LIGHT

High-intensity laser or noncoherent light has long been known to be capable of inactivating microorganisms,[78] though it is often unclear to what extent the effects seen result from the ultraviolet component of the irradiation and, sometimes, local transient heating. Low power ruby lasers were shown to inactivate vegetative bacteria more than 30 years ago[66] and later studies demonstrated the killing effects of laser light at wavelengths from 193 nm in the ultraviolet to 10,600 nm in the infrared.[1,31,97,128]

A number of studies explored the use of lasers to inactivate microorganisms in the medical area, particularly dentistry.[19,97,103] The delivery of light to equipment surfaces, packaging material, food product surfaces, and transparent liquid foods, in short duration high-intensity pulses, has been described and shown capable of inactivating vegetative and spore forms of microorganisms in these environments.[24] The numbers of *E. coli* in biofilms adhering to stainless steel surfaces were reduced more than 10^3-fold by a single 20 nsec pulse of laser light delivered at less than 50 MW (Megawatts) cm^{-2}. Energy densities up to 600 MW cm^{-2} resulted in more than 10^6-fold reductions.[104] It was shown that the biofilmed surface was decontaminated primarily by physical removal ("ablation") rather than by thermal or photochemical inactivation of the bacteria. Efficiency was, therefore, increased when the aerosolized organisms released by the process were drawn off to minimize redeposition. The process was more effective than most commonly used chemical treatments, which generally deliver 2- to 3-log inactivation of vegetative bacteria in biofilms on a variety of materials, including stainless steel.[8,52] While safety and technical constraints may limit the widespread use of pulsed lasers for equipment surface decontamination in the near future, dedicated uses, such as fixed in-line components of equipment requiring decontamination at critical points, are more likely, for example, to decontaminate rotating blades in cooked meat slicing processes.[104]

Surface treatments with 1,064 nm light at an output pulse energy of about 10 J delivered over 8 msec at a pulse repetition rate of 10 Hz inactivated a variety of Gram-negative and Gram-positive bacteria (*E. coli, Serratia marcescens, S. aureus, B. subtilis, B.*

stearothermophilus, Micrococcus luteus, Deinococcus radiodurans) and yeasts (*S. cerevisiae, C. albicans*). Using a rough endpoint method of assessment (growth/no growth from initial numbers of probably 10^6 or so), *C. albicans* was the most sensitive, inactivated by 1,388 J cm^{-2}; *E. coli* and *M. luteus* were of intermediate resistance (2,020 and 3,130 J cm^{-2}, respectively) and vegetative cells of *B. stearothermophilus* were most resistant (4,371 J cm^{-2}). Total treatment times were in the order of 20 seconds.[126]

Commercially practical machines for treating foods with high-intensity noncoherent light pulses, coupled with aseptic packaging, have been patented.[24] These machines use broad spectrum light with pulse durations from 10^{-6} to 10^{-1} second and with energy densities from 0.1 to 50 J cm^{-2}. Different spectral distributions and energies are selected to match the requirements of different applications. For example, ultraviolet-rich light in which about 30% of the energy is at wavelengths shorter than 300 nm is recommended for treatment of packaging materials, water, or other transparent fluids. The killing effects in this case mainly derive from the ultraviolet component of the absorbed light. In contrast, for the treatment of food surfaces, particularly when ultraviolet irradiation may cause undesirable changes in the product, such as acceleration of lipid oxidation or color bleaching, the ultraviolet is filtered out. The killing effects are then largely thermal. The advantage of delivering heat in this manner is that a large amount of thermal energy is transferred to a very thin layer of the food surface very quickly. Consequently, the temperature rise in the interior of the food is very small.[24] The efficacy of the machines have been demonstrated against vegetative cells (*E. coli, S. aureus*), yeast (*S. cerevisiae*), and spores of bacteria (*B. subtilis*) and fungi (*Aspergillus niger*). The contribution of the ultraviolet component was illustrated by the much reduced killing effect of ultraviolet-filtered light. While one pulse of 12 J cm^{-2} unfiltered light inactivated vegetative cells of *B. subtilis* by about 6 logs, as many as 15 pulses of ultraviolet-filtered light only reduced numbers by about 0.5 log, and 25 pulses by about 3.5 log.[24]

Overall, therefore, these intense light treatments are effective mainly because they deliver conventional inactivating treatments, ultraviolet irradiation or heat, but in an unconventional manner that may be advantageous in some circumstances.[78]

13.5 HIGH-INTENSITY MAGNETIC FIELDS

Exposure to oscillating magnetic fields has been claimed to have a variety of effects on biological systems ranging from selective inactivation of malignant cells with relatively little damage to normal tissues[20] to the inactivation of bacteria on packaging materials and in foods.[50] The antimicrobial procedures involve exposing the target materials to pulses of very high magnetic intensity, at magnetic fluxes from 2 to about 100 tesla, at frequencies between 5 and 500 kHz. Total treatment time is very short, typically from 25 µsec to a few msec.

The mechanism of action is not known. Temperature rise during magnetic pulse treatments was not more than 5°C, and usually about 2°C. It has been suggested that the magnetic energy couples into dipolar molecules and may change membrane fluidity and alter ion fluxes across cell membranes.[96] No inactivation of bacterial spores has been reported. Efficacies of the treatments did not exceed about 100-fold reductions in numbers of vegetative microorganisms inoculated into milk (*Streptococcus thermophilis*), orange juice (*Saccharomyces*), and bread rolls (unspecified mold spores),[50] therefore, the practical applications for the technique appear to be limited.[5,78]

13.6 MANOTHERMOSONICATION

The use of ultrasound to accelerate chemical reactions and to inactivate microorganisms was first proposed 70 years ago.[42] Since then, ultrasound has been employed widely in *sonochemistry,* for cleaning and for dispersing, homogenizing, and emulsifying liquids, and has been promoted for speeding up age-related changes in foods such as meat and wine.[105]

The mechanism of action of ultrasound derives from the rapidly alternating compression and decompression zones propagating into the material being treated and the cavitation that these cause. Cavitation involves the formation and collapse of small bubbles, generating shock waves of very high local pressure and temperature that can be sufficiently intense to catalyze chemical reactions and to disrupt animal, plant, and microbial cells.[112] Generally, large cells are more susceptible than small ones.[2] Cocci are more resistant than rod-shaped bacteria[3] and Gram-positive more sensitive than Gram-negative bacteria,[2] while spores are so much more resistant as to be essentially nondisruptable.[109]

Early work on the biocidal effects of ultrasound was usually carried out with temperature control designed to rule out any effects due to heat. It was not until 1972 that deliberate determination of the combined effects of ultrasound and heat showed that a potentially useful synergy occurred, even with the normally ultrasound-resistant spores of *B. cereus* and *B. licheniformis*,[9] as well as with thermoduric streptococci,[87] *S. aureus*, and other vegetative microorganisms.[86] Further work confirmed the heat-ultrasound synergism, but also showed that as the temperature was raised the potentiating effect of ultrasound became less and less, disappearing at the boiling point of the menstruum, ie, near to 100°C for most foods.[33] The important discovery that led to the development of manothermosonication (MTS) was that this disappearance of the synergism could be prevented by raising the pressure.[105] MTS therefore involves simultaneously heating and ultrasonicating foods while under a small overpressure, commonly no more than 1 MPa (about 10 atmospheres). The process generally has the effect of reducing the apparent heat resistance of microorganisms by about 5° to 20°C or, on a kinetic basis, by between 6- and 30-fold depending on the temperature, the organism, and its *z*-value. Yeasts are more sensitive than bacteria to the additional effects of ultrasonication. Figure 13-8 illustrates the magnitude of the effects of MTS on spores. While the MTS process has not yet been commercialized, it has been shown to operate in liquid foods (eg, milk[105]), offering the possibility of new sterilization or pasteurization processes for this and other liquid foods, such as fruit juices, sauces, and dressings, with reduced levels of thermal damage. It is less likely that the process would be of value for solid foods or foods containing other than very small particulates because of the absorption of the ultrasonic energy that would be expected to occur.

13.7 CONCLUDING REMARKS

The use of physical techniques to inactivate microorganisms in foods without the application of heat, or with the delivery of less heat than would otherwise be necessary, is attractive from the point of view of food product quality. The techniques surveyed above all aim to achieve this. However, four principal factors limit their usefulness at present.

The first is that bacterial spores remain the organisms most resistant to all the new techniques, so that their use for the sterilization of low acid–high a_w foods for long ambient stability is not possible, with the possible exception of MTS. Consequently, the techniques are limited to pasteurization-like processes for foods in which spores are not a problem (eg, jams and fruit juices) or which have a limited chill shelf life. Effective control of spores by combining the new techniques with other preservation adjuncts has already been proposed (eg, pressure plus heat, pressure and electric pulse treatments plus bacteriocins, the MTS treatment itself) and may offer a way forward, but the commercial feasibility of such approaches remains to be established.

The second important factor is that, should such combination treatments be pursued, then their safety implications require much more consideration. For example, in the use of alternative methods for the commercial sterilization of low acid–high a_w foods (eg, low-acid canned foods) the same safety factor that is used in heat processing, ie, a 12-decimal reduction of viable spores of *C. botulinum*, would be needed. How-

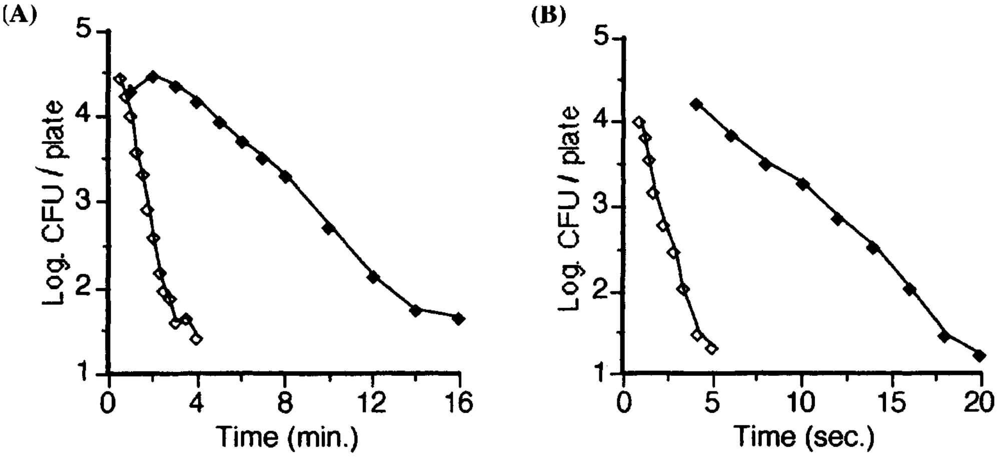

Figure 13-8 Synergism of Heat with Ultrasound (20 kHz, 117 μm) under Pressure (300 KPa) in the Inactivation of Spores of *Bacillus subtilis*. MTS-treated (◇) and heated controls (◆). **(A)** 100°C. **(B)** 112°C. *Source:* Reprinted from F.J. Sala et al., Effect of Heat and Ultrasound on Microorganisms and Enzymes, in *New Methods of Food Preservation,* G.W. Gould, ed., pp. 176–204, © 1995, Aspen Publishers, Inc.

ever, spores of some strains of *C. perfringens, B. cereus, B. subtilis,* or *B. licheniformis* may show greater resistance (relative to that of *C. botulinum* spores) to alternative processes than to heat and may therefore contribute to a need for a more severe treatment than dictated by *C. botulinum* spores. In the development of heat pasteurization processes the main pathogens against which the processes were targeted were (1) for pasteurization of milk, *Mycobacterium* and *Brucella* species and *Coxiella burnetii* (2) for liquid egg, *Salmonella* species (3) for "Sous Vide" and similar foods, vegetative pathogens, and spores of nonproteolytic *C. botulinum.* New methods of pasteurization should be designed to give at least an equivalent safety factor to that delivered by heat processing against these organisms. In addition, it should be recognized that other vegetative pathogens may show a higher degree of resistance to the alternative methods of processing than do the organisms mentioned above; for example, *L. monocytogenes* and verotoxin-producing *E. coli* (in particular *E. coli* O157) may need particular consideration. It would be necessary to select the relevant pathogen that showed the highest resistance to the proposed type of treatment and to specify that the extent of treatment given should reduce its viable numbers by an agreed factor, eg, 10^4, 10^5, or 10^6.

Third, the kinetics of microbial inactivation by some of the techniques is evidently far from the exponential relationship that, even with the deviations sometimes known to occur, has become the basis of sound thermal processing. It may be difficult to accommodate unusual kinetics, eg, into sensible risk analyses for the new processes.

Fourth, although an advantage of the most successful of the new processes, isostatic pressure, is that it treats all parts of a food equally, the efficacy of most of the other techniques is impaired by food structure. In particular, high-intensity light is limited to relatively transparent foods or surfaces, electric pulses and MTS will probably be limited to liquid foods or foods with small particulates.

However, while it is important to be clear about the possible problems, there clearly are opportunities for the use of some of the new techniques and, particularly if new combination approaches are developed and proven to be effective, these opportunities are likely to expand in the future.[4]

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14

Use of Combined Preservative Factors in Foods of Developing Countries

Lothar Leistner

CHAPTER CONTENTS

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14.1 INTRODUCTION

The preservation of almost all foods in industrialized and developing countries is based on combinations of several preservative factors that secure microbial safety, stability, and the sensory quality of these foods. This is true not only for traditional foods, but also for novel products. The most important preservative factors (also called hurdles) in common use for food preservation are high temperature (F value), low temperature (t value), water activity (a_w), acidity (pH), redox potential (Eh), some preservatives, and the competitive flora.[42] However, more than 50 factors (hurdles) of potential use for improving the stability and quality of foods have been described,[12] and the list of possible hurdles for the preservation of foods is by no means complete.

The factors used in combination for the preservation of foods might have additive or even synergistic effects on each other. The complex interactions of preservative factors present in foods is illustrated by the so-called hurdle effect,[34,42] which indicates that a food is stable and safe if the hurdles in this food cannot be overcome by the microorganisms present. From an understanding of the hurdle effect, hurdle technology has been derived,[36] which allows improvements in the microbial stability and sensory quality of foods by deliberate and intentional combinations of hurdles. Over the years, the insight into the hurdle effect has been broadened and the application of hurdle technology extended. In industrialized countries, the hurdle technology concept is currently of particular interest for minimally processed, fresh-like foods. In developing countries the application of hurdle technology is most attractive for the preservation of foods that are stable and safe without refrigeration.

The application of hurdle technology is advancing rapidly worldwide. This concept is synonymously called Hürden-Technologie in German, hurdle technology in English, technologie des barrières in French, barjernaja technologija in Russian, tecnologia degli ostacoli in Italian, tecnologia de obstaculos in Spanish, shogai gijutsu in Japanese, zang lan gi shu in Chinese, etc.[48] However, it should be noted that the hurdle effect has been used empirically since ancient times. It was used for the mummification of the Egyptian pharaohs as an old example of "tissue preservation" based on the hurdle effect. Mummies contain at least three hurdles, namely reduced a_w (0.72), increased pH (10.6), and preservatives (spices, aromatic plants), as has been demonstrated by Chirife et al.[16] However, only recently has increased understanding of the principles behind the hurdle effect made it feasible to begin to replace empirical processes by an intentional and intelligent selection of hurdles for the preservation of foods by the application of hurdle technology.

This chapter will concentrate on foods of developing countries. First, the principles of food preservation procedures used in developing countries will be discussed. Secondly, some recent developments in food preservation based on hurdle technology in different regions of the world will be outlined, by discussing examples from Latin America, China, India, and Africa.

14.2 PRINCIPLES OF FOOD PRESERVATION IN DEVELOPING COUNTRIES

There exists an abundant variety of preserved foods in developing countries because the gap between harvest peaks has to be bridged, and the taste and nutritional properties of foods might improve because of preservation. The processes employed for the preservation of foods in developing countries are largely based on the same principles as in industrialized countries, such as heating, chilling, freezing, drying, salting, sugar addition, acidification, fermentation, oxygen removal, and addition of preservatives.

However, the emphasis differs considerably. Whereas in industrialized countries, low temperatures (chilling, freezing) are widely used for the preservation of food, the required energy for these processes is costly in many developing countries and not reliably available. Canning and autoclaving, ie, the application of high temperatures, are also less suitable for developing countries because they require large investments and much energy. On the other hand, reduced water activity (a_w) is a suitable and prevalent procedure for food preservation in developing countries. Methods of drying and/or addition of salt or sugar to foodstuffs are often combined with a reduced pH (increased acidification). Fermentation of foods also is a traditional and common preservation process in developing countries. If lactic acid bacteria are used for fermentation, acidification (low pH) contributes to the preservation of foods. If molds are employed, in addition or separately, the taste and the nutritional properties of foods during fermentation improves. The variety of fermented foods is larger in developing than in industrialized countries, and the types of products differ considerably by the regions. Oxygen removal by vacuum packaging is already quite widespread in developing countries, an older process with a similar purpose is to cover foods with a layer of oil. Preservatives are occasionally added in excessive amounts to foods by artisans in developing countries, but in some regions, such as some parts of Africa, they might be too expensive and therefore "natural preservatives," like spices or herbs and their extracts, are preferred. For other reasons industrialized countries prefer to avoid chemical preservatives, and also prefer conventional or unconventional natural preservatives.

It may be concluded that reduction of the water activity is the principal preservative factor in developing countries, and that intermediate-moisture foods (IMF) with an a_w range of 0.90 to 0.60 are the prevalent food items. However, practically none of the food manufacturers in developing countries has the possibility to measure a_w, and few even recognize the relevance and significance of the water activity for the preservation of their foods.

Within the framework of the CYTED-D program (Science and Technology for Development, Fifth Centennial), which was sponsored by Spain and to which Argentina, Brazil, Chile, Costa Rica, Cuba, Mexico, Nicaragua, Puerto Rico, Uruguay, and Venezuela have contributed, two projects were carried out from 1986 to 1994.[77] The first project, entitled "Development of Intermediate Moisture Foods (IMF) Important to Ibero-America" had the objectives of identifying and evaluating foods of Latin America that are storable without refrigeration. The foods comprised fruits, vegetables, and bakery products as well as foods derived from fish, milk, meat, and miscellaneous products. About 260 food items were approved to be microbiologically stable and safe without refrigeration. The properties (a_w, pH, preservatives, food composition, etc) and the production technology for these products were described.[1,76] It was concluded that most of the approved products were intermediate-moisture foods. However, some had high a_w values and nevertheless were stable and safe without refrigeration, and it turned out that the stability of these high-moisture foods was caused by a combination of several empirically applied hurdles. This observation was the starting point for Latin American researchers to apply intentional hurdle technology[36] to high-moisture foods, especially fruits, storable without refrigeration. Therefore, the second project within the framework of the CYTED-D program, with national financial contributions from the participating countries, was entitled "Bulk Preservation of Fruits by Combined Methods." The main objective of this project was to develop simple and inexpensive processes that would help to diversify the Latin American fruit industry and to reduce the large post-harvest fruit losses.[77] These two projects of the CYTED-D program resulted in an impressive number of refereed papers, contributions to food congresses, and B.S. and graduate theses.

The Latin American CYTED-D study demonstrated a promising approach for improving the stability of foods in developing countries, which could also be applied in other regions. Following this concept, first the properties of already available, microbiologically stable and safe food items should be thoroughly studied and described. Second, the preservation factors (hurdles) effective in these foods, and thus the principles behind their microbial stability and safety, must be elucidated. Third, if feasible, the preservation and quality of these foods should be improved by the intentional application of hurdle technology.

Using this concept, Tapia de Daza et al.[64] have identified the hurdles in the food items studied within the first project of the CYTED-D program, and by critical evaluation of the hurdles that have been traditionally applied to certain foods, they assessed the products' microbial stability and safety. It was demonstrated that similar hurdles are active in the same type of foods in different Latin American countries. However, there were also surprising differences, which pointed to an over- or underprocessing of the same food in different countries of the same region. This insight could lead to the avoidance of some nonessential preservatives or, on the other hand, to an improved stability, safety, and quality of some food items by fortification of certain hurdles.

In the following sections, the principles on which the preservation of intermediate-moisture and high-moisture foods in developing countries are based will be discussed in relation to the preservative factors (hurdles) applied.

14.2.1 Intermediate-Moisture Foods

Latin American researchers[64,76] pointed out that reduced water activity was the main hurdle in the intermediate-moisture foods (IMF) they described within the first CYTED-D project. However, in practically all of these products, additional hurdles were present that contributed considerably to stability and safety. Thus, the microbial stability of most of these IMF was based on a combination of preservation factors. This observation confirmed the opinion expressed by Leistner and Rödel[33] that in most intermediate-moisture foods several hurdles are inherent.

A case in point is pastirma, a typical IMF of developing countries. Pastirma is a traditional beef product of Moslem countries. It is storable for several months at ambient temperatures and is eaten in the raw state.[19] The stability and safety of pastirma is based on the reduced a_w (0.90–0.85), in combination with several additional preservative factors.[38] The interior of this product is stabilized by the addition of salt and nitrate (which is reduced by bacteria to nitrite) and the removal of water by drying and pressing of the meat, as well as by the growth of lactic acid bacteria, which decreases the pH to about 5.5. These hurdles secure the inhibition of spoilage and pathogenic bacteria, including salmonellae, within the meat. The surface of pastirma is covered with an edible paste containing 35% freshly ground garlic and other spices (paprika, kammon, mustard with fenugreek as a binder). This surface coating prevents the growth of molds on the product during storage, even at higher humidities and temperatures. Therefore, at least five hurdles (a_w, nitrite, pH, competitive flora, garlic) are relevant to the preservation of pastirma. Guidelines suggested for safe processing of traditional pastirma (Exhibit 14-1) shall secure that the hurdles inherent to this product are effective.[38]

Tapia de Daza et al.[64] listed many traditional IMF of Latin America, derived from fruits, vegetables, meat, milk, fish, etc, in which several identified

Exhibit 14-1 Guidelines for Processing of Traditional Pastirma, a Raw Meat Product of Moslem Countries

1. Lean meat from the hindquarter of healthy beef cattle should be used (this secures low initial bacterial counts).
2. Meat should be cut into strips of not more than 5 cm in diameter (allows a quick penetration and equilibration of salt).
3. The a_w of the meat must be decreased quickly by addition of sufficient salt and by pressing the meat to reduce moisture (the salt content of the finished product should be 4.5% to 6.0% and the a_w 0.90–0.85).
4. With the salt about 600 ppm nitrate is added to the meat (which after reduction to nitrite inhibits undesirable bacteria, especially at reduced pH).
5. Growth of lactic acid bacteria (up to 10^7/g) in the paste and in the meat is desirable as competitive flora and for pH reduction (but for sensory reasons the pH should not be <5.5).
6. After drying, the meat must be covered with a paste containing several spices and 35% freshly ground garlic (the spices and especially the garlic inhibit salmonellae in the meat as well as mold growth on the surface of the product).

Source: Adapted with permission from L. Leistner, Shelf-Stable Products and Intermediate Moisture Foods Based on Meat, in *Water Activity: Theory and Applications to Food,* L.B. Rockland and L.R. Beuchat, eds., p. 322, © 1987, by courtesy of Marcel Dekker, Inc.

hurdles contributed to microbial stability and safety. Very often the reduced a_w was combined with a reduced pH. However, in some salted fish and shrimps, the pH was >8.0, and thus in these foods the elevated pH might contribute to preservation. Many meat, fish, fruit, and dairy products contained additional preservatives (eg, nitrite, smoke, benzoate, sorbate, sulfites, spices, and Maillard products) and sometimes competitive microorganisms. Maillard reaction products, which are generated during the desired caramelization of sweet condensed milk (dulce de leche), are probably an important hurdle for this Latin American food item.

A special case concerns candied fruits common in this region. Their heavy sugar coating acts as a physical barrier (hurdle) against microbial contamination after the heat process, so that these foods are stable during storage in spite of the absence of preservatives and a sometimes rather high pH. Quite often a thermal treatment is used to inactivate heat-sensitive microorganisms during the manufacturing process and to improve microbial stability, and a vacuum in sealed containers is achieved by hot filling of the products. Once the container is opened, the redox potential increases and then the microbial stability of these IMF against molds and yeasts must be secured by the preservatives present. Additional hurdles are employed in particular in those IMF which have rather high a_w and/or pH values. In the IMF studied, it was exceptional if the low a_w was apparently the only hurdle present. This was likely in a dried Brazilian meat product as well as in traditional salted fish with an a_w close to 0.75. However, in most IMF products, three to five hurdles have been identified.[64]

How many hurdles, besides the a_w, are active in intermediate-moisture foods depends on the type of the product; however, it may be concluded that the microbial stability and safety of IMF in general is based on a combination of several preservative factors.

14.2.2 High-Moisture Foods

Many food products with a water activity above 0.90 need refrigeration during storage, and therefore are less suitable for developing countries. For example, cooked ham with much water added (a_w close to 0.99) was introduced to China, but such products easily spoil or even cause food poisoning if not adequately chilled. Therefore, developing countries should be cautious when introducing high-moisture foods from industrialized countries.

However, there are Western meat products available that, in spite of a rather high water activity (a_w 0.97–0.92), are microbiologically stable and safe without refrigeration because they have been stabilized by the application of intentional hurdle technology.[44,48] The same holds true for other food items that are indigenous to developing countries,[1,64,76] even though their effective preservative factors (hurdles) have only been partially identified.

There is a trend in developing countries to move gradually away from intermediate-moisture foods because IMF are often too salty or too sweet, and have a less appealing texture and appearance than high-moisture foods (HMF). High-moisture fruit products (HMFP) were studied in the second project of the CYTED-D program.[77] It might be expected that high-moisture, fresh-like foods, which are storable without refrigeration because they are stabilized by combined preservative factors, will be generally on the increase in developing countries as soon as the application of hurdle technology has been mastered. Such products are minimally processed, convenience foods, now the trend in industrialized as well as in developing countries. These foods are often refrigerated in industrialized countries; however, in developing countries they should remain stable, safe, and even fresh-like if stored for an extended time at ambient temperatures.[66,67] The independence from the chill chain renders high-moisture, convenience foods, such as HMFP, much more suitable for developing countries.[6]

14.3 RECENT APPLICATIONS OF HURDLE TECHNOLOGY IN DEVELOPING COUNTRIES

Food preservation in developing countries has a time-honored tradition and a wealth of products are known which deserve respect.[40] Nevertheless, improvements in microbial stability, safety, and sensory quality of these foods are feasible. Researchers in developing countries are working in this direction by taking recent accomplishments in food science and technology into consideration. One of these developments is the knowledge about food preservation based on an understanding of the hurdle effect and the application of hurdle technology. This knowledge emerged from the German Meat Research Institute and a number of relevant publications[33,35,36,38,42,44,45,48,49,50] were utilized by food scientists worldwide. In addition, the author of this chapter was invited in recent years to present numerous lectures and courses in more than 20 developing countries.

Presently, several groups of researchers are working worldwide on the improvement of traditional foods as well as the development of novel products, by taking the hurdle effect and hurdle technology into account. Some examples of recent results obtained in Latin America, China, India, and Africa will be discussed.

14.3.1 Latin America

Fruits

In general, fruits are an important and abundant commodity in developing countries; therefore, Latin American researchers have concentrated on improvements in the preservation of tropical and subtropical fruits. They first concluded from their studies that intermediate-moisture fruits (IMF) are more attractive for consumers than fully dried products.[76] However, the high solute concentration necessary to depress the water activity of IMF often causes undesirable alterations of the product flavor. Therefore their second conclusion was that intermediate-moisture fruits do not seem to be a good option for tropical and subtropical fruit marketing.[77] Jayaraman from India agreed with these conclusions in a recent review paper, and pointed out that intermediate-moisture fruits face technological problems and consumer health concerns associated with the high levels of humectants and preservatives required for their shelf stability.[30,31]

Therefore, work was launched in Latin America to preserve fruits as high-moisture foods (HMF) by using combined preservative factors (hurdle technology). Objectives of this approach were to develop simple, inexpensive, energy efficient processes for the local fruit industry, which could overcome seasonal production constraints and reduce post-harvest losses (Exhibit 14-2). The main goal was to preserve fruits in fresh-like condition even if stored for several months without refrigeration.[66,67] The first success in this endeavor was reported with preservation of peaches.[62] By using a similar approach, an excellent microbial stability and fresh-like quality was achieved with pineapple[3] as well as with banana, chicozapote, mango, and papaya,[4] and in an independent study again with papaya.[53] Additional investigations added figs, passion fruit, plum, pomalaca, strawberry, and tamarind to the list of fruits preserved as high-moisture products.[5] The fruits were preserved whole, sliced, or as purees. For some fruits, the process was scaled up to a pilot plant–size operation. However, the introduction of these processes to real industrial production has not yet been achieved.[67]

Exhibit 14-2 Objectives for Preservation of Fruits in Bulk by Combined Methods in Latin America

To develop technologies that should:
—be simple and inexpensive
—be energy efficient (independent of chill chain)
—be suitable for processing at production sites
—help overcome seasonal production constraints
—contribute to diversification of local fruit industries
—help to reduce post-harvest losses
—preserve fresh-like properties of raw materials

Source: Adapted with permission from M.S. Tapia de Daza, S.M. Alzamora, and J. Welti Chanes, Combination of Preservation Factors Applied to Minimal Processing of Foods, *Critical Reviews in Food Science and Nutrition*, Vol. 36, pp. 629–659, © 1996, CRC Press, Inc.

Within the CYTED-D program, seven Latin American countries contributed to this exercise, and outstanding contributions came from Argentina (Departamento de Industrias, Universidad de Buenos Aires, Buenos Aires), Mexico (Departamento de Ingeniería Química y de Alimentos, Universidad de las Américas-Puebla, Puebla), and Venezuela (Instituto de Ciencia y Tecnología de Alimentos, Universidad Central de Venezuela, Caracas).

Alzamora et al.[3,4] have suggested five hurdles for the preservation of high-moisture fruit products (HMFP): (1) mild heat treatment (to inactivate enzymes and to lower the initial microbial load), (2) slight reduction of a_w (by addition of sucrose or glucose), (3) pH adjustment if necessary (by addition of citric or phosphoric acid to fruits with higher pH), and (4) and (5) the addition of two preservatives (potassium sorbate or sodium benzoate and sodium sulfite or sodium bisulfite) in moderate amounts (Table 14-1).

The process is quite simple to carry out (Figure 14-1) and, therefore, could be established at harvesting

Table 14-1 Typical Process Used for the Preservation of High-Moisture Fruit Products (HMFP)

Hurdles	*Intensity*
1. Heat treatment*	Saturated steam for 1–3 minutes
2. Water activity†	a_w reduction to 0.98–0.94
3. Acidification‡	pH adjustment to 3.0–4.1
4. Preservative (I)	1,000 ppm sorbate or benzoate
5. Preservative (II)	150 ppm sulfite or bisulfite

*Dependent on size and type of fruit.
†Adjusted with sucrose, glucose, maltodextrins, etc.
‡Adjusted with phosphoric or citric acid.
Source: Data from [3, 4, 5, 7, 66].

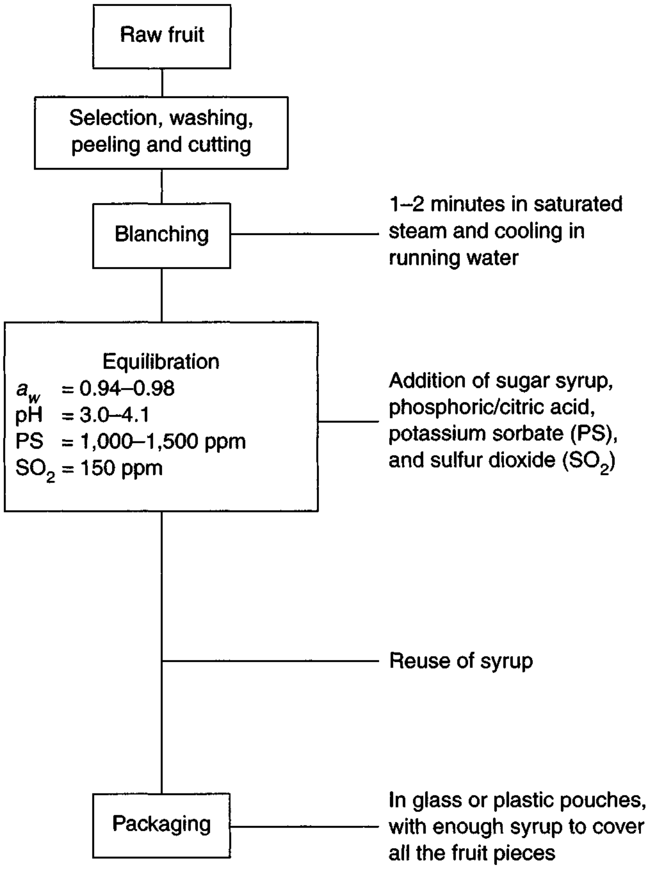
Raw fruit
Selection, washing, peeling and cutting
Blanching
1–2 minutes in saturated steam and cooling in running water
Equilibration
a_w = 0.94–0.98
pH = 3.0–4.1
PS = 1,000–1,500 ppm
SO_2 = 150 ppm
Addition of sugar syrup, phosphoric/citric acid, potassium sorbate (PS), and sulfur dioxide (SO_2)
Reuse of syrup
Packaging
In glass or plastic pouches, with enough syrup to cover all the fruit pieces

Figure 14-1 Flow Diagram for the Production of Shelf-Stable HMFP (Mango, Papaya, Peach, Pineapple, etc). *Source:* Adapted with permission from M.S. Tapia de Daza, S.M. Alzamora, and J. Welti Chanes, Combination of Preservation Factors Applied to Minimal Processing of Foods, *Critical Reviews in Food Science and Nutrition,* Vol. 36, pp. 629–659, © 1996, CRC Press, Inc.

places. It results in stable and safe fresh-like products storable for at least 3 to 8 months (ie, from one harvest peak to the next) at ambient temperatures by using modest packaging.[3,4,7,53] Even after this long storage at 25°–35°C, sometimes in bulk, the taste, flavor, color, and texture as well as the overall acceptability were rated by untrained panels (30 to 50 members) quite high,[4,5,53] as shown in Table 14-2. This is especially noteworthy for the texture, which often is not satisfactory after canning or freezing of fruits. The main characteristics lost and, therefore, the ones that determine the sensory shelf life of HMFP are color and flavor.[7] The average scores of HMFP have always corresponded to products with good acceptability, which leads to the conclusion that such products can be regarded as attractive alternatives to conventional fruit preservation methods.[4,7,53,66,67] Preserved HMFP can be eaten as they are or may be used for out-of-season processing in confectionery, bakery goods, dairy products, jams, jellies, etc. Fruit pieces can also be utilized as ingredients in salads, barbecues, pizza, yogurt, and fruit drink formulations.[5,7] Because HMFP can help to reduce post-harvest losses and serve as a supply regulator, they may lead to important economical and social impacts.[7]

The effective preservative factors listed in Table 14-3 have proven suitable to secure the microbial stability and sensory quality of HMFP. However, these hurdles should not be regarded as inflexible, and if other combinations of preservative factors become known that are even more suitable for the stability and quality of HMFP, they should be used instead.[7,66,67] Argaiz et al. pointed out that by a modification of the types or levels of hurdles used in HMFP the sensory quality of these products could be further improved because the present hurdles were selected primarily with respect to microbial stablity.[7] For instance, a combination of citric and phosphoric acids or a total substitution of citric by

Table 14-2 Sensory Properties of Shelf-Stable HMFP (Mango, Papaya, Peach, Pineapple) after Long Storage at Ambient Temperatures

Attribute	*Average Score**
Taste	6.65–7.70
Odor	5.80–6.80
Texture	6.70–8.07
Color	6.46–7.10
Overall acceptability	6.73–7.63

*Obtained by untrained panels (30 to 50 members) using a 9-point hedonic scale (9, like extremely; 1, dislike extremely), after storage (30 to 152 days) of HMFP at ambient temperatures (25° to 35°C).

Source: Adapted with permission from M.S. Tapia de Daza, S.M. Alzamora, and J. Welti Chanes, Combination of Preservation Factors Applied to Minimal Processing of Foods, *Critical Reviews in Food Science and Nutrition*, Vol. 36, pp. 629–659, © 1996, CRC Press, Inc.

phosphoric acid in the preservation of peach by combined methods enhanced their sensory acceptability; however, this was not the case if sucrose was replaced by glucose as water activity depressor.[52] Furthermore, sulfites are effective as browning inhibitors and antimicrobials in fruits, but there are health concerns and therefore alternative browning inhibitors should be investigated.[7,55]

The preparation steps to which fresh fruits are subjected in the production process for HMFP (Figure 14-1), will have an impact on the natural flora of fruits because some procedures will decrease and others increase the microbial load. While washing may remove many of the surface organisms, the peeling, cutting or slicing might contaminate the fruit flesh. Blanching is mainly employed to inactivate enzymes in vegetables and fruits, however, it also inactivates many heat-sensitive microorganisms. Reductions in the microbial load due to blanching from 60% to 99% have been reported.[3,5,53] In addition, microorganisms that survive the heat treatment during blanching might become sensitized to other hurdles. A further decrease in viable numbers is expected to occur during the equilibrium stage in which the blanched fruit pieces or purees are exposed to the stress effects of a_w, pH, and preservatives.[65] During storage the surviving bacteria, yeasts, and mold in the syrup and the fruits of HMFP generally decrease rapidly, and the final products become practically sterile.[3,4,5,27,53,62,65] However, this occurs only if all hurdles listed in Table 14-1 are present.[27] Table 14-3 gives examples of the rapid decrease of the counts of bacteria, yeasts, and molds during storage of some HMFP, which have been studied by several groups in Latin American countries.[3,53,65] Probably the major decrease of the microbial counts in HMFP occurs already during the equilibration phase, which requires about 3 to 5 days at ambient temperatures.[66,67] However, microbiological data of this critical phase are not yet available. Because HMFP during storage become apparently sterile, pathogenic and toxigenic microorganisms are not likely to be a hazard for these foods.

Table 14-3 Microbial Counts (cfu g^{-1})* Present in Mango, Papaya, Peach, and Pineapple Preserved As HMFP and Stored for Several Months at Ambient Temperature

	Days of Storage				
Type of HMFP	*0*	*15*	*30*	*50*	*120*
Mango (slices)	22	<10	<10	<10	<10
Papaya† (slices)	1,230	100	<10	<10	<10
Papaya† (slices)	<10	<10	<10	<10	<10
Peach† (halves)	1,000	300	100	<10	<10
Peach† (halves)	<10	<10	<10	<10	—
Pineapple (rings)	6	<10	<1	<2	<1

*Mesophilic, aerobic bacteria plus yeasts and molds.
†Data from different studies.
Source: Data from [3, 53, 65].

To observe the actual behavior of microorganisms in HMFP, it is essential to use appropriate diluents and detection or enumeration media for assessing accurately the microbiological status of hurdle technology foods.[9,10,65] The microbial stability and safety of HMFP should not only be tested by following the kinetics of the decrease of the "natural" microbial flora during processing and storage, but also by conducting challenge tests with appropriate species and numbers of microorganisms.[65] Guerrero et al.[27] performed challenge tests in a high-moisture banana puree, by using strains of *Zygosaccharomyces bailii, Z. rouxii, Saccharomyces cerevisiae, Aspergillus niger, Eurotium amstelodami, Penicillium roquefortii, Paecylomyces varioti, Bacillus coagulans, Clostridium butyricum*, and *C. pasteurianum*. The banana puree supported no growth of the organisms tested if the following hurdles were present: a_w 0.97, pH 3.4, addition of 100 ppm potassium sorbate, 400 ppm sodium bisulfite, and 250 ppm ascorbic acid.[27]

The added sorbate in the preserved HMFP degrades somewhat during storage, depending on the time and temperature of storage as well as on pH, a_w, light, presence of oxygen, packaging material, etc.[3,6,22,23,53] For instance, in peaches, pineapples, and papaya preserved by the HMFP method, sorbic acid destruction during storage amounts to about 40%.

Sulfites degrade in HMFP even faster and more completely than sorbate, depending on the type of food and the length and conditions of storage.[3,7,53,67] If the free sulfite concentration decreases too much, undesirable browning of the fruits might occur. The depletion of sorbate and sulfites during storage of HMFP is favorable in view of preservative intake by the consumer; however, it diminishes the microbial stability of the products. On the other hand, in HMFP, during storage, hydrolysis of sucrose to fructose and glucose occurs; this results in a slight decrease of the water activity of the products,[17,56] which improves the microbial stability. Generally, the interaction between applied hurdles and the food matrix must be carefully considered.[6]

Chemical preservatives, such as sorbate and sulfites, are viewed with suspicion also in developing countries, and "natural" preservatives, such as spices and herbs, are more appealing to the public. However, the addition of antimicrobial spices in effective levels is often limited by sensory impairment of the foods. This is less critical for vanillin because the taste of this major constituent of vanilla beans is compatible with the taste of various fruits.[5] It is noteworthy that vanillin used to flavor foods often originates not from vanilla beans but is a by-product of the paper industry, and thus it is not really "natural." The inhibitory effect of vanillin against yeasts[14] and molds[54] was investigated with the intention to use this "natural" preservative in HMFP. The results suggested that vanillin is of potential use for preventing yeast and mold spoilage of fruits (except those with high fat and/or protein contents, like banana and mango), if combined with a slight reduction of a_w and pH. Cerrutti et al.[15] reported that 3,000 ppm vanillin was effective to achieve microbial stability of strawberry puree, if combined with a mild heat treatment (blanching), addition of 500 ppm ascorbic acid and 1,000 ppm calcium lactate, and adjustment of a_w to 0.95 and pH to 3.0. These hurdles prevented the growth of native and inoculated spoilage flora (yeasts, molds, bacteria) for >60 days at room temperature (about 23°C).[15] However, vanillin has to be used in higher concentrations and is more expensive than the common preservatives.[54]

The fast and apparently complete disappearance of bacteria, yeasts, and molds in HMFP during processing and storage deserves particular attention. The applied blanching process (heating for 1 to 3 minutes with saturated steam) considerably reduces the microbial load of the fruits. This is an important prerequisite for the success of the following preservation steps,[5,27,65] because if fewer microorganisms are present "at the start" not so many and not so high hurdles are needed to inhibit their growth.[42] It is noteworthy that the heat treatment (blanching) in the presence of preservative hurdles would be much more effective than without them.[27] The number of bacteria, yeasts, and molds that survive the blanching (some become heat injured) and the equilibration process decreases quite rapidly within days and often below the detection limit within a few weeks of storage of the HMFP.[3,27,53,65] This surprising autosterilization of fruits preserved by the HMFP process is most likely due to *metabolic exhaustion* of the microorganisms present[47] because microorganisms that have used up their energy are not able to repair their disturbed homeostasis.[24,26] Because in the HMFP process several preservative factors (a_w, pH, sorbate, sulfite) are active, probably different targets within the microbial cells are hit at the same time, and due to this *multitarget preservation*[47] a synergistic effect of the applied hurdles is likely.[25,26] Fundamental studies about the physiological basis of the growth, survival, and death of microorganisms during food preservation are desirable, which, in the case of HMFP, for example, could underpin the observed autosterilization of the preserved foods with a better understanding of the underlying principles. In these studies, homeostasis, metabolic exhaustion, and stress reactions of microorganisms as well as the multitarget preservation of foods should be taken into account, as proposed by Gould[24,25,26] and Leistner.[47,48] The significance of stress reactions of microorganisms in hurdle technology foods, which could be compensated by multitarget preservation of these foods,[47] was recognized by Alzamora et al.[6] These Latin American researchers concluded that "less processing of foods requires more hurdles," and this definitely applies to minimally processed foods including HMFP.[6] However, these additional hurdles must certainly be compatible with the nutritional soundness and sensory quaility of minimally processed foods.

In spite of effective hurdles in HMFP, resistant microorganisms might cause spoilage problems. Of practical importance is that some yeasts develop resistance against the preservative factors acting in HMFP. *Zygosaccharomyces bailii* is especially of concern with HMFP, because this yeast might exhibit a considerable resistance to heat, low pH, reduced a_w, sorbic and benzoic acid, and sulfite.[68] It was demonstrated that an apparently resistant strain of *Z. bailii* did survive and grow in inoculated mango (a_w 0.97, pH 3.5) and papaya (a_w 0.98, pH 3.5) with an addition of 150 ppm sodium sulfite and 1,000 ppm potassium sorbate, after the sorbate level had decreased during storage of the products below about 600 ppm and the sulfite was depleted.[65] Therefore, the critical levels of preservatives for the growth of *Z. bailii* should be established and monitored during storage of HMFP.[66] Additional yeast species, such as *Z. rouxii*, *Saccharomyces acidifaciens*, and *S. bisporus* are also of concern because they might develop resistance to sorbic and benzoic acids.[9,58] *Z. bailii* and *Z. rouxii* have been recommended to be used for challenge tests conducted during the design of HMFP.[67] The probability that resistant yeasts occur in HMFP should be reduced by proper sanitary procedures and guidelines for the processing of HMFP.[66,67]

The preservation of HMFP must certainly be based on guidelines for Good Manufacturing Practice (GMP) or, if feasible and necessary, on Hazard Analysis Critical Control Point (HACCP), in order to be successful under industrial or even artisan conditions.[49] For instance, the reuse of the syrup of HMFP could become a risk in relation to a build-up of preservative-resistant spoilage yeasts, and therefore, a reuse of syrup should only be recommended after pasteurization. Furthermore, the containers used for bulk and long storage of HMFP should be covered with a lid in order to avoid a recontamination of HMFP after the added preservatives have depleted. Suitable GMP rules must be defined. Because pathogenic and toxinogenic bacteria are apparently not a hazard for HMFP, especially due to the low pH as well as to the other hurdles hitherto applied,[7,66,70] the introduction of HACCP plans seems less appropriate than GMP rules for HMFP.

Guidelines are also useful for the design and the development of HMFP. For this purpose, Tapia de Daza et al. have recommended a 13-step procedure,[66,67] which resembles the 10-step guideline suggested for the design of hurdle technology foods by Leistner.[46] The user guide to design of high-moisture fruit products by hurdle technology as proposed by Tapia de Daza et al.[65,66,67] is outlined in Exhibit 14-3. Well-designed HMFP should lead to an autosterilization of the products during storage at ambient temperatures, by using mild but effective hurdles that secure microbial safety and stability as well as the sensory quality of HMFP.

Exhibit 14-3 User Guide to Design of High-Moisture Fruit Products (HMFP) by Combined Method

1. Proposal of the sensory properties, shelf life, packaging, and storage requirements of the desired product.
2. Suggestion of a tentative process technology for this HMFP.
3. Consideration of the properties inherent to the raw material:
   a. Microflora usually associated with this particular fruit
   b. Physical, biochemical, and chemical properties of this fruit
4. Outline of a preliminary process:
   a. Screening of the potential hurdles
   b. Performance of the potential process
   c. Predictive microbiology used in preliminary stability testing
5. Definition of prototype process based on results obtained in 4:
   a. Determination of process parameters
   b. Identification of hazards and weak process points
6. Manufacturing of the product according to process of 5:
   a. Microbial evaluation of the control points
   b. Evaluation of the final product properties
7. Storage studies (sensory, physical-chemical, microbial tests).
8. Isolation/identification of resistant microflora in HMFP.
9. Execution of challenge tests using relevant microorganisms.
10. If modifications of the hurdles are necessary start again with 6.
11. If modifications not necessary, final definition of the process.
12. Scaling-up of the applied process under industrial conditions.
13. Control of the industrial process according to GMP or HACCP.

Source: Adapted with permission from M.S. Tapia de Daza, S.M. Alzamora, and J. Welti Chanes, Combination of Preservation Factors Applied to Minimal Processing of Foods, *Critical Reviews in Food Science and Nutrition*, Vol. 36, pp. 629–659, © 1996, CRC Press, Inc.

Alzamora et al.[5] pointed out that the HMFP technology as developed in Latin America might give rise to an "explosion" of studies and applications of this innovative approach for fruit preservation in many developing countries. In this respect it is noteworthy that a preliminary study conducted in India already confirmed the results obtained in Latin America with HMFP. The Indian researchers concluded that "hurdle technology is seen as a promising technique for the preservation of fresh fruits and vegetables."[61]

Fish

The concept of hurdle technology is certainly also applicable for the preservation of fish; however, experimental work in this field is until now scanty. In several Latin American countries, fish is an important and often underutilized commodity as human food, and thus, interest in improved preservation methods is strong.

In Chile (Departamento de Ingeniería Química, Pontificia Universidad Católica de Chile, Santiago), the use of combined methods to extend the shelf life of sardines (*Sardinops sagax*) was studied by Aguilera et al.[2] Sardines and other pelagic fish from the western coast of South America are used mostly as raw material in fish meal production for animal feed. Their large-scale utilization as human food demands low-cost and simple processing and storage techniques. Therefore, the use of combined preservative factors was explored. The shelf life of minced sardines was extended from less than 3 days for the original unwashed mince to at least 15 days at 15°C by the following process: washing, addition of 6% sodium chloride (to obtain an a_w of 0.94) and 0.2% potassium sorbate, reduction of pH to 5.7 with acetic acid, packaging, and heat treatment of the product in a waterbath at 80°C for 2 minutes at the geometric center. The total count of the stabilized minced product remained very low during the whole storage period. Mincing of headed and gutted underutilized fish is considered to be an approriate process for their use as food. However, the mince of sardines, due to the light and dark tissues of this fish, is not attractive and therefore must be washed four times in order to remove dark pigments and the high fat content of sardines, and also to lower the microbial load by a factor of 100. The rather high salt content proved necessary for microbial stability, and the reduced pH contributed to inhibition of microbial growth primarily by increasing the effectiveness of sorbate. The mild heat treatment results in only minor changes in the textural and functional properties of the mince. The proposed hurdle technology food is considered to be an important alternative to traditional drying and/or salting, which stabilize the products but dramatically change the organoleptic properties of fish.[2]

In Argentina (Centro de Investigaciones de Tecnología Pesquera y Alimentos Regionales [CITEP], Mar del Plata), the application of hurdle technology for the preservation of fish was initiated at the beginning of the 1990s by Yeannes and coworkers, and several reports and papers on this subject have been published in Spanish or English since then.

Work was conducted with mackerel (*Scomber japonicus marplatensis*), anchovy (*Engraulis anchoita*), and salmon (*Pinguipes somnambulus*), in relation to the microbiology of the marination process, the microbiology and quality of intermediate- or high-moisture fish products, and the use of stabilized fish paste for making hamburgers, paté, etc. The results are applicable to fat and lean fish species, ie, mackerel and salmon, respectively. Some of these products are already on the market, for example, slices or chunks of salmon stabilized by salt, natural smoke, vinegar, potassium sorbate, and vacuum packaging, which are storable at least for 30 days at chill temperatures (Ing. Maria I. Yeannes, 1995; personal communication).

Two of the published research papers will be discussed. The microbiology of the marination process to be used for pelagic anchovy was studied by Fuselli et al.[21] The authors investigated different types of raw material (fresh, presalted, frozen), and each stage of an experimental marination process (sampling after washing the fillets, immersion into brine, immersion into a marinating bath, and packaging with acetic acid solution or oil), with the aim to extend the diversity of products made from anchovies. The results indicated that all types of raw material were suitable for marinating, and that the marination process (based on the combined preservative action of sodium chloride and acetic acid) was suitable to control the growth of pathogenic and spoilage bacteria, and thus might lead to a microbiologically stable product of good sensory quality. Because some bacteria survive the marination process, their behavior during the storage of the products was further investigated and will be reported later.[21]

Sanchez Pascua et al[63] studied the experimental development of an intermediate-moisture mackerel product with microbial stability based on the reduction of the water activity (caused by the addition of sodium chloride and glycerol), the addition of potassium sorbate, and a mild heat treatment. The fish was cooked for 20 minutes in the infusion solution (containing salt, glycerol, and sorbate) and afterwards was immersed in this solution for 20 hours at 2°C. The white muscle of the fish, with a_w of 0.89 and pH of 6.5, was packaged in glass jars with vegetable oil and pasteurized at 90°C for 40 minutes. The product obtained by this combined preservation process had a moderately sweet taste and a succulent texture; a bitter taste was not perceived. Therefore, such a product might be well accepted by the consumer. In future microbiological studies the shelf life of this product will be evaluated.[63]

Both of the quoted studies[21,63] from Argentina are of a preliminary nature. However, they indicate clearly that the application of combined factors might be useful for the preservation of fish in developing countries.

In Peru (Instituto Tecnologica Pesquero del Peru, Callao), a project on "Hurdle technology empirically applied in ancient and modern Peru" commenced (Dr. Guy Carvajal and Ing. Maria E. Ayala, 1996; personal communication). First, it was documented that in pre-Columbian times salting of fish was often used in combination with drying and smoking, as vestiges found at coastal Peruvian sites have attested. Still today, in the high plains of Peru, where the humidities are low, small whole fish are dried by traditional procedures. Another procedure used in ancient Peru for the preservation of food was the use of plants or essential oils from plants. During the time of the Incas, fish was covered with mint (*Mintostachys mollis*), a wild plant frequently encountered in the Peruvian Andean mountains, which has antibacterial and weak antioxidant properties. With this cover it was possible to transport fish without spoilage for two days at ambient temperatures. However, the mint extracts have been proven to be mutagenic.[13]

In recent decades other hurdles have been introduced, especially chilling (with ice) during transportation and storage, as well as heading and gutting of fish, which diminishes the bacterial load. In a novel process, the fish flesh (pulp) of sardines (*Sardinops sagax sagax*), horse-mackerel (*Trachurus symmetricus murphy*), or mackerel (*Scomber japonicus marplatensis*) is acidified with 0.5% to 2% citric acid and salted to contain 24.5% sodium chloride (a_w 0.75). After vacuum packaging, this product has a shelf life of at least three months at ambient temperatures. In order to delay rancidity of the lipids, the fish today is often dipped in antioxidants (BHA, tocopherol, etc) prior to salting or the antoxidants are added to the brine. Antioxidant-treated sardines may be stored with acceptable quality for one month at room temperature.

Another method used for preservation is fermentation. In Peru, silage for animal feed is obtained from cooked mince of waste fish, inoculated with yogurt bacteria and sugar cane is used as carbon source. A sugar concentration of 5% to 10% is enough to achieve a pH of 4.0 within two days at 40°C. Histamines slightly increased during incubation but decreased again during storage. The silage is stable and safe for at least 180 days at ambient temperatures, having an aromatic odor, which resembles fermented fruits. In Peru, work has been initiated to apply the hurdles mentioned as well as others systematically for the preservation of fish for human consumption (Dr. Guy Carvajal and Ing. Maria E. Ayala, 1996; personal communication).

Meat

In Latin America, a number of traditional intermediate-moisture meat products are common, eg, of the 260 foods studied in the course of the CYTED-D projects, 29 were meat products.[1] With the intention to improve the sensory quality of some of these meats, modifications based on combined preservative factors are at present under investigation.

In Brazil at the Universidade de Sao Paulo (USP) and the Universidade Estadual de Campinas (UNICAMP), experimental work is in progress to modify charqui, a typical Brazilian intermediate moisture meat product and an important source of animal protein, particularly for rural people in regions where refrigeration facilities are limited.[11,69] Charqui consists of large flat pieces of meat, mainly from the flank or forequarters of beef, preserved by salting and sun drying. The raw meat with a uniform thickness of 2–4 cm is submerged in a saturated salt solution for about one hour, and thereafter dry-salted in piles. To form a pile, marine salt is spread evenly on a concrete floor and then a layer of meat is put on the salt. The meat is covered with another (1 cm) layer of salt followed by another layer of meat, and so on until the alternate layers of salt and meat reach a height of about one meter. After eight hours, the pile is restacked so that the top meat goes to the bottom of the pile. The restacking process with fresh layers of salt is repeated every day for five days. Thereafter, the meat pieces are subjected to rapid washing to remove excess salt adhering to the surface. Now the meat is exposed to the sun on wooden rails for four to five days. At night, meat pieces are collected and stacked in piles on a concrete floor and covered with a tarpaulin.[40,57] Therefore, charqui is preserved by salting and drying, and it contains about 45% moisture and 15% sodium chloride, which results in an a_w of 0.70–0.75.[11,69] This traditional meat product is storable for several months without refrigeration. Charqui has a typical strong flavor and a slightly rancid taste. Fermentation during the drying phase, possibly caused by *Pediococcus halophilus*, might contribute to the typical flavor and as a hurdle to the preservation of charqui; however, this fermentation process is not well understood. On the other hand, it is known that halophilic bacteria might spoil (sour) the product.[40] If raw material of high quality is used, a gradual decrease of microbial counts during the processing and storage of charqui might occur. Thus, final products with low microbial levels are obtained if good raw materials and adequate handling conditions are used for charqui production and storage.[69] Charqui is always eaten in the cooked state. Full rehydration of the product before consumption is not possible even after prolonged soaking, but excess salt might be removed in this manner. The partially desalted charqui is often consumed as an ingredient for a typical Brazilian dish known as "feijoada," which contains black beans or rice and other desalted dried organs of beef or pork carcasses.[57,69]

The appearance, flavor, taste, and high salt content of traditional charqui today are not appealing to every consumer anymore, and therefore a derivative known as carne bovina salgada curada seca or jerked beef[11] was recently introduced to the market. As raw material for this product, meat from the forequarters of beef is stiched-pumped with a brine containing salt and nitrite, followed by dry curing and drying, and vacuum packaging of the finished product. Thus, the microbial stability and safety of jerked beef as well as traditional charqui is based on hurdle technology.[11] However, whereas in charqui, besides the a_w hurdle, a possible *Pediococcus* fermentation is only a minor hurdle; in jerked beef more hurdles have been introduced, resulting in sensory quality improvements. However, the hurdles necessary for the stability and quality of jerked beef are still regarded as too intense. Further investigations, also with similar products, are at the present time being conducted at the Universidad Estadual de Campinas (Prof. Bento da Costa Carvalho Júnior, 1996; personal communication).

14.3.2 China

China has a wealth of traditional foods, the recipes of many of those date back centuries. Moreover, China out of necessity and for culinary reasons makes use of a huge variety of raw materials as sources for delicious dishes and preserved food products. The microbial stability and safety of almost all of the latter are based on combined preservative factors empirically applied. However, because intentional hurdle technology (zang lan gi shu, in Chinese) was introduced by the author of this chapter, who is a permanent consultant to the Chinese Meat Research Center located in Beijing, interest in this innovative concept has grown. Some examples of recent applications of hurdle technology to meat products in China are subsequently discussed.

Meat

Work related to the application of hurdle technology on meat products is currently carried out in several Chinese research institutes (eg, Sichuan Provincial Research Institute of Animal and Veterinary Science, Chengdu; China Meat Research Center, Beijing; Hangzhou Institue of Commerce, Hangzhou; Heinaer Institute for Export Control, Inner Mongolia). Several recent publications in Chinese[51,73,74,75,82,83] have outlined the concept of hurdle technology, and thus made it popular in the People's Republic of China.

The per capita consumption of meat, compared to Western countries, is rather low in China. Nevertheless, because of the huge number of people living in China, this country has the highest meat consumption worldwide. Pork is the preferred meat in China; however, beef, water buffalo, sheep, duck, chicken, goose, rabbit, donkey, dog, and other species are used as raw materials.[40]

Only about 15% of the available meat of China is processed.[71] This is a large amount, and meat products are an important and precious part of the diet. There are two categories of meat products existing in China side by side: Chinese meats and Western meats. Western-type meat products were introduced to China during the last four decades. They are attractive because of their high yield, nice appearance, and pleasant taste. However, they still cause difficulties with respect to storage and microbial shelf life, because in China refrigeration is scarce and costly. Therefore, especially in the summer months, and if the microbial load of the raw material is too high or the hygienic conditions during meat processing are not adequate, spoilage and considerable economic losses frequently occur.[82] On the other hand, the traditional Chinese meat products are prevalent in China[71,72] because they are quite simple to prepare without expensive equipment, have a typical flavor, are ready-to-eat, and are storable without refrigeration. Some of the classical Chinese meat products, known for centuries, are listed in Table 14-4. The microbial stability and sensory quality of all of the products are based on combined preservative factors. Studies are in progress to identify the hurdles inherent to these traditional meats,[72] and thus an optimization of those products, if desirable, becomes feasible by the application of hurdle technology.[73]

The traditional Chinese meat products listed in Table 14-4 are all intermediate-moisture foods (a_w range 0.90–0.60). This implies that they might be too salty or too sweet, too tough and too dark due to the formation of Maillard reaction products. The water activity is the primary hurdle in these meats, but if additional hurdles are strengthened, then the a_w might be raised, which often improves the sensory

Table 14-4 Traditional Chinese Meat Products

Cured meats (Yan La)	Chinese bacon—La Rou Pressed duck—Ban Ya Silk rabbit—Cha Si Tu Cured chicken—Yuan Bao Ji
Dried meats (Rou Gan)	Dried meat—Rou Gan Sweet meat—Rou Pu Meat floss—Rou Song
Sausage (La Chang)	Guangdong La Chang, Sichuan La Chang, etc.
Raw ham (Ho Tui)	Yü Nam Ho Tui, Jin-Hua Ho Tui, etc.

Note: All of these meat products are intermediate-moisture foods and are storable without refrigeration.

Source: Data from W. Wang and L. Leistner, Traditionelle Fleischerzeugnisse von China und deren Optimierung durch Hürden-Technologie, *Fleischwirtschaft*, Vol. 74, pp. 1134–1145, © 1994.

quality of the products. However, the microbial stability and safety of the meats must not be jeopardized by an increased a_w. Therefore, intentional and intelligent hurdle technology is increasingly applied. Some examples of such endeavors are cited below.

Rou gan, a typical dried meat product of China, is prepared mainly from beef but also from mutton or pork, using a technology that has not been changed for hundreds of years, but improvements are possible and desirable. Consumers now prefer products with a softer texture, lighter color, and less sugar addition. Shafu is a modified dried meat that fulfills these expectations. The a_w of rou gan is in the range of 0.60–0.69, whereas shafu has an a_w of 0.74–0.76 because its moisture is higher and the sugar content lower. Additional hurdles in shafu are nitrite and ascorbic acid, which improve the color and delay rancidity, whereas rou gan is cured with nitrate only. Furthermore, the microbial stability of shafu is improved by the selection of raw material with low microbial load, low temperature during curing but relative high temperatures and shorter times during heating, and prevention of recontamination after the heat process as well as vacuum packaging of the final product in order to inhibit mold growth and to delay rancidity. Therefore, shafu is microbiological stable and safe for several months at ambient temperatures, and thus has the same shelf life as the traditional rou gan.[71]

A similar approach was chosen to improve the quality of Islamic dried beef in dices, which is also storable for several months without refrigeration. The traditional product is just salted and dried, and therefore is very salty and becomes easily rancid. The modified product contains less salt, is cured with nitrate to improve the color, and some ascorbic acid is added to delay rancidity. Furthermore, vacuum packaging is applied to reduce oxidation and to avoid mold growth.[78] These additional hurdles are very similar to those in modified charqui of Brazil, ie, jerked beef, discussed above.

Basic studies on the color,[79] flavor,[80] and taste,[81] using up-to-date methodology, have been conducted in China on the famous jin-hua ho tui. This is a traditional raw ham (Table 14-4) known for more than 800 years in China, and is much appreciated for its red muscle, white fat, characteristic flavor and taste, and the long shelf life.[79,80,81] Jin-hua ham resembles similar products of Europe (eg, prosciutto di parma of Italy, jamón serrano of Spain), and of the United States (eg, Virginia ham, country cured ham). All these bone-in hams are produced with a similar technology, cured with sodium chloride only and ripened under carefully controlled climatic conditions. They are characterized by a processing time of several months, which leads to the typical flavor (see Chapter 19). The microbial stability and safety as well as the quality of these raw hams are based on combined preservative factors, ie, a sequence of hurdles, which have been empirically applied to these products for centuries, but are today better understood.[37,38] Exhibit 14-4 presents guidelines for the production of traditional bone-in hams with a technology and preservative factors (hurdles) quite similar for products processed in Europe, the United States, or China.

Apparently the delicious flavor and the bright color of these hams are mainly caused by the activity of indigenous enzymes of the meat, without much participation of bacteria, yeasts, or molds. However, undesirable bacteria might lead to spoilage (which is most often caused by psychrotolerant Enterobacteriaceae) or even to food poisoning (caused by nonproteolytic *Clostridium botulinum* type B), if the green (fresh) hams harbor too many bacteria, have a pH >5.8, and are not kept <5°C until in all parts a NaCl content of >4.5% is achieved, which corresponds to a_w of 0.96. The finished products should contain not more than 5% to 7% NaCl.[37,38]

Presently, the NaCl content (8% to 15%) of jin-hua ham and similar products of other provinces of China is unnecessarily high with respect to safety and is detrimental to product quality.[72,73] Therefore, investigations are in progress to produce hams that contain less than 4% salt. However, with respect to the microbiological stability and safety, it is hazardous to lower the salt content of jin-hua ham below

Exhibit 14-4 Guidelines for the Processing of Traditional Bone-in Raw Hams of Europe, United States, and Asia

1. Careful transport and slaughtering of hogs (secures low initial counts of bacteria in the interior of the hams).
2. Chilling of hams in 24 hours to <4°C in the core (inhibits rapid growth of bacteria inside the hams).
3. Hams with pH >5.8 are excluded from raw ham production (thus meat with poor curing and high spoilage properties is eliminated).
4. Curing temperature in the first three months shall not exceed 5°C (inhibits bacteria inside hams during the curing process).
5. Hams are cured until in all parts a_w is <0.960; corresponds to >4.5% NaCl (inhibits bacterial growth in hams during ripening).
6. Ripening time is 7 to 20 months at 20°–25°C (30°C) (secures full flavor of finished products, which shall contain 5% to 7% NaCl).

Source: Data from L. Leistner, Allgemeines über Rohschinken, *Fleischwirtschaft,* Vol. 66, pp. 496–510, © 1986, and L. Leistner, Shelf-Stable Products and Intermediate Moisture Foods Based on Meat, in *Water Activity: Theory and Applications to Food,* L.B. Rockland and L.R. Beuchat, eds., pp. 295–327, © 1987, Marcel Dekker, Inc.

5%, and to shorten the curing time at low temperature. Moreover, a shortcut of the subsequent ripening time at ambient temperatures might jeopardize the flavor of the products. Jin-hua ham, like other long ripened bone-in hams of Europe and America, is definitely a hurdle technology food product. The primary hurdle in these hams is the water activity. However, this hurdle becomes only slowly effective due to salt penetration and drying. Therefore, the initial bacterial counts in the hams must be low and growth of bacteria must be prevented by an initially low pH and strict refrigeration, until a critical a_w has been reached in all parts of the ham. Only then is it possible to raise the temperature, in order to speed up enzymatic ripening and flavor development of the product. The finished hams have an increased pH due to desired proteolysis, but the a_w is at this stage sufficiently low to secure a stable and safe product. Chinese researchers are now aware of the significant hurdles in jin-hua hams, and will take them into consideration in the course of their studies related to lowering the salt content and shortening the curing as well as the ripening time of jin-hua ho tui (Assoc. Prof. Shangwu Zhu, 1996, Hangzhou Institute of Commerce; personal communication).

A procedure that can be considered as an unusual variant of hurdle technology is now applied in the processing of frozen meat for export in such remote places as Mongolia.[51] This has improved the quality of expensive meat cuts from exotic wild animals (eg, Mongolian gazelles), by taking the following hurdles into account: initial microbial counts of the meat, water activity, redox potential, and temperature. Based on these hurdles an efficient process has been designed. The carcasses of shot animals arrive frozen at the meat plant, are cleaned, and the meat is frozen again. Freezing reduces the a_w and the microbial counts. For cutting, the meat is only partially defrosted; this avoids an increase of microbial counts as well as moist meat surfaces. This operation is carried out quickly under hygienic conditions. Then the meat with a temperature around 0°C is vacuum packaged, with careful exclusion of air in order to achieve a low Eh in the packages. Finally, the cuts are quick frozen at –25°C and shipped below –18°C without delay. The implementation of this process has much improved the microbial and sensory quality of such frozen cuts.[51] Therefore, this is a nice example of the practical and successful application of hurdle technology to frozen meat of China.

In Taiwan (Department of Food Science, Taichung University, Taichung), in cooperation with the German Meat Research Institute, hurdle technology was applied to stabilize la chang.[32] This Chinese sausage is highly esteemed by Oriental consumers, and differs from the fermented sausages common in Western countries because it is also processed raw, however, with only little fermentation. Several types of la chang (also called la zang or lup cheong) are known, eg, the Sichuan type is more spicy and the Cantonese type sweeter, whereas the Taiwanese la chang is softer. In mainland China, finished products of la chang have the following properties: a_w 0.85–0.70, pH 5.9–5.7, salt 3% to 5%, sugar 4% to 20%, total count $<10^6$ g^{-1}, and shelf life two to three months without refrigeration and, if vacuum packaged, even four to five months. To the coarsely ground meat and fat from pork, sugar, salt, soya sauce, liquor, spices, nitrate (or nitrite), and sometimes sodium ascorbate are added. The mix is stuffed into casings with small diameter, and the sausages are dried fast over charcoal at 45°–60°C to a_w of 0.92. They are then further dried at ambient temperature to a_w <0.80 in order to avoid an increase of the lactic acid bacteria counts because generally the Chinese dislike sour meats. Thus, la chang is a typical meat product preserved by combined factors. In general, before consumption la chang is sliced and heated in rice or vegetable dishes.[32,38,72,73] Angkak is often added to Chinese sausage. This is rice fermented with *Monascus* spp., which contains red pigments as well as flavoring and antimicrobial metabolites. The pigments are heat, light, and pH stable, whereas the antimicrobial substances inhibit definitely Gram-positive and moderately also Gram-negative bacteria,[41,43] and thus angkak could be considered to be an additional hurdle applied in la chang.

The Taiwanese variety of Chinese sausage contains more moisture, and therefore can have an a_w as high as 0.94. This improves the sensory properties because the products are softer. However, it decreases their microbial stability and safety because in such products lactic acid bacteria still grow and lead to a sour taste, and *Staphylococcus aureus* might cause food poisoning. After addition of 3.5% sodium lactate and 0.1% sodium acetate, the Taiwanese sausage remains tasty but is rendered microbiologically stable and safe, even if stored for several weeks without refrigeration. These additives reduce the a_w of the products but also have some antimicrobial effects.[32] Challenge tests using inocula of *Staphylococcus aureus*, *Listeria monocytogenes*, or *Salmonella* spp. confirmed these effects, and demonstrated that during storage at 25°C, the number of pathogens decreased faster than at 10°C.[32] The latter was probably due to a faster metabolic exhaustion of the pathogens at the higher storage temperature.[47]

Chinese meat technologies are also employed in other Asian countries, such as Singapore, Malaysia, and the Philippines, but with local variations. Also, in Western countries with Asian minorities, Chinese meat products are increasingly on the market. In general, for developing countries the traditional Chinese meats as well as Chinese meat products optimized by intelligent hurdle technology should be of interest, especially if meat products must be stored without refrigeration. Because Chinese meat technology is based on different principles, but also results in safe, stable, and high quality products, it might be a source of innovation for Western countries. On the other hand, novel hurdle technology meats developed in Western countries have been already implemented in China. For example, shelf-stable heated meat products of the F-SSP type[36,44] are now produced in large quantities in the Luoyang city and other regions of China.

14.3.3 India

Lectures in India by the author of this chapter on food preservation using combined preservative factors, as well as reports on this subject published by Indian scientists[8,29,60,61] have stimulated research about the application of hurdle technology to traditional and modified Indian food products. Work related to food preservation using hurdle technology is at present carried out at several research institutes of India (eg, Central Food Technological Research Institute (CFTRI), Mysore; BHABHA Atomic Research Centre, Food Technology Division, Trombay (Bombay); Indian Institute of Technology, Bombay; University of North Bengal, Siliguri; National Dairy Research Institute (NDRI), Karnal).

Dairy Products

In India, vegetarian food is common, but dairy products are also much appreciated. Some recent examples of the application of hurdle technology to Indian dairy products are discussed.

Paneer is a traditional, cottage cheese–type product fried in cubes with oil and onions, to which a sauce containing salt, spices, and often tomatoes is added. This food is frequently consumed and much liked in the Northern provinces of India because of its nutritive value and characteristic taste. However, paneer spoils bacteriologically within one to two days at room temperature (which in India can reach 40°C), which is a strong drawback for its industrial production. Sterilized paneer in cans has severe sensory limitations with regard to flavor, texture, and color. Therefore, together with a visiting scientist from India, Dr. K. Jayaraj Rao, in the German Meat Research Institute, Kulmbach, a mildly heated paneer in hermetically sealed containers, with the desired flavor (like prepared fresh), color (little browning), and texture (not too hard) was developed.[59] This product was stabilized by hurdle technology, and thus is stable and safe for several weeks without refrigeration. The following combinations of hurdles proved effective with this product: a_w = 0.97, heating to F_0 value of 0.8, pH = 5.0 or alternatively a_w = 0.96, F_0 = 0.4, pH = 5.0.[59] After his return to India, Rao conducted a thorough study on the application of hurdle technology to fried paneer in cubes made of buffalo milk. The product with gravy was packed either in tins or flexible retort pouches, and a set of hurdles, ie, a_w = 0.95, F_0 = 0.8, pH = 5.0, and 0.1% potassium sorbate, was chosen, which had maximum lethal and inhibitory effects on microorganisms and minimum effects on textural and chemical characteristics.[60] The water activity of paneer and gravy was lowered by using humectants, such as *dahi*, skim milk powder, salt, and glycerol. The pH was adjusted by changing the *dahi*/skim milk powder ratio. The resulting product had a keeping quality of two weeks at 45°C, one month at 30°C, or over three months at 15°C, which was limited by textural changes (hardness, cohesiveness, gumminess, springiness, and chewiness) as well as by chemical changes (browning, oxidation, lipolysis, and loss of available lysine), but not by microbiological spoilage. A mathematical shelf life prediction model based on sensory scores and quality parameters was developed. The stabilized product was compared with fresh samples from restaurants and was found to be equally acceptable. In the opinion of Rao,[60] this method of paneer preservation has a large scope for alterations in product formulations depending on regional taste preferences, without affecting the keeping quality of the product. Via paneer, the hurdle technology was introduced into food science of India, and its application to other indigenous foods is now anticipated.

A recent example is dudh churpi, a popular dairy product of the Himalaya region of India (ie, Bhutan, Sikkim, Darjeeling), made of milk from yaks or cows. This product is stable for several months without refrigeration. Most important for dudh churpi is the texture (elasticity), because people living in high altitudes chew it as an *energy tablet*. The sensory quality and microbial stability of dudh churpi was optimized by Hossain[29] using combined methods (hurdle technology) of heating, acid coagulation, addition of sugar and sorbate, smoking, drying, and packaging in closed containers. In this detailed and diligent study, dudh churpi was scientifically explored and a feasible optimization of the product was suggested. Thus, hurdle technology was applied to improve a traditional food of the remote Himalayan region. At the same time, the scientific basis of this study opened new avenues for food science and industrial food production in India. Hurdle technology is perceived now by some Indian scientists as the most appropriate technology for preserving foods in developing countries (G.R. Patil, 1992; personal communication).

14.3.4 Africa

The author of this chapter introduced the application of hurdle technology for the preservation of shelf-stable foods during an invited lecture presented in 1988 at a meeting of the South African Association for Food Science and Technology.[39] Since that time this concept was successfully applied to various food products, especially by Dr. Alexander von Holy and his group (Department of Microbiology, University of Witwatersrand, Johannesburg). A recent example is the prevention of spoilage (ropiness) of bread that results from the growth of amylolytic *Bacillus* species, which causes significant losses to the baking industry in South African conditions. By the addition of calcium propionate in combination with vinegar to the dough, reducing the spore counts in the raw materials and on the equipment, and temperature control during and after baking, it was possible to avoid the ropiness of bread. This is considered to be a practical application of food preservation using combined methods.[28] In another recent study on the spoilage of vacuum packaged Vienna sausages resulting from the growth of lactic acid bacteria during storage, it was investigated whether an in-package pasteurization of the products at about 60°C could delay spoilage. This was indeed possible if the products were stored at 8°C. However, the pasteurization processes studied decreased the occurrence of leuconostocs and homofermentative lactobacilli more than pediococci, and increased the incidence of bacilli and clostridia. The presence of *Clostridium perfringens* in pasteurized samples indicated that in-package pasteurization of Vienna sausages could compromise product safety if the temperature was allowed to rise.[20] Since the political situation in South Africa has changed, it is anticipated that food science and technology of neighboring African states will be fostered by cooperation with South Africa. Thus, the application of combined methods (hurdle technology) for food preservation will probably now spread.

However, in some African countries researchers are already aware and interested in the application of hurdle technology for the preservation of foods. For instance, Dr. Bernard O. Ejechi and his group (Department of Microbiology, Delta State University, Abraka, Nigeria) are working on the preservation of various food items by means of combined methods.

Tomatoes, which are abundant in Nigeria only at certain times of the year, are often minced with salt in the home, boiled, covered with a thin layer of groundnut or palm oil, and then stored for several weeks at ambient temperatures because refrigeration is too expensive. This traditional hurdle technology food has the drawback that the supply of oil is scarce because it is needed for cooking. Therefore, oil should be replaced by preservatives that the average consumer can afford. Because nutmeg, vanilla, and pepperfruit seeds are readily available in Nigeria, the preservative effects of their aqueous extracts were investigated. In addition, the sodium chloride addition and the heat treatment of the tomato blend, in relation to the sensory quality of the product, were standardized. By using tomato-rot fungi (*Candida* species, *Saccharomyces* spp., *Geotrichum* spp., and *Rhizopus* spp.) for challenge tests, it was established that by the following hurdle combination a shelf life of three months at room temperature of the tomato blend and a good sensory quality of the product were achieved: addition of 2% sodium chloride and 1% aqueous extract of pepperfruit (*Dennetia tripetala*) seeds, and a heat treatment at 60°C for 30 minutes.[18]

Furthermore, the Nigerian researchers accomplished a shelf life of three months of orange and pineapple juice with the following hurdle combination: addition of spice extracts from nutmeg, vanilla, or ginger and heat treatment at 70°C for 5 minutes. However, color changes of the juices caused by the spices are undesirable; therefore, sodium metabisulfite was added in small amounts, which allows for reducing the level of added spice extracts. With mango juice, a shelf life of three months was achieved, after addition of aqueous extracts (4%) of spices (ginger, nutmeg) combined with a mild heat treatment (50°C for 15 minutes), and in this case the sensory quality of the juice with the added spice extracts was acceptable (Dr. B.O. Ejechi, 1996; personal communication).

In Nigeria soya bean milk is increasingly used as baby food, especially among the low-income groups because conventional baby food is expensive. Therefore, the microbial stabilization of this easily perishable food is also an issue. The hurdles considered for preservation of soya bean milk are the addition of lemon juice (5%) and sodium chloride (1%) combined with a mild heat treatment (50°C for 5 minutes), or the addition of aqueous ginger extract to improve the flavor and possibly the shelf life of this food (Dr. B.O. Ejechi, 1996; personal communication).

Thus, combined methods were explored recently for the preservation of various foods in Nigeria, and it is noteworthy that "natural" preservatives, like spice extracts, are preferred to chemical preservatives because they are readily available and less expensive.

14.4 CONCLUDING REMARKS

The microbial stability and safety as well as the quality of almost all foods in developing countries is based on a combination of preservative factors (hurdles). Such preservation processes have been applied empirically since time immemorial to traditional foods, which are often intermediate-moisture foods (a_w range 0.90–0.60), and storable without refrigeration. Of course, refrigeration is known in developing countries; however, energy is very expensive and not continuously available and, therefore, foods microbiologically stable and safe without refrigeration are much preferred.

In developing countries the hurdles are chosen intentionally because of better understanding of the hurdle effect, and thus, the preservation of foods is optimized by the application of hurdle technology. Such foods are often in the high-moisture range (a_w above 0.90) and have an improved sensory quality, but are storable without refrigeration. It is a general trend in developing countries to move gradually away from intermediate-moisture foods because they are often too salty or too sweet, and have a less appealing texture and appearance than high-moisture foods. Thus, it is expected that fresh-like, high-moisture foods, which are nevertheless storable without refrigeration, will be on the increase in developing countries as soon as the application of hurdle technology has been mastered. In the opinion of an Indian food scientist, the most appropriate technology for preserving foods in developing countries is the application of hurdle technology.

However, use of hurdle technology should not lead to the addition of high levels of additives, but actually should reduce the amounts of additives used even if their number might increase. It is important that additional hurdles be introduced into a food product only after careful consideration of the necessity and in essential amounts, otherwise an undesirable chemical overloading of the food could result. Under artisan conditions especially, there is sometimes an indiscriminate use of chemical food additives, eg, sulfite to fruits or nitrate to meats, and such shortcomings must be prevented in modified hurdle technology foods. If possible, "natural" additives such as spices or herbs and their extracts should be employed because they are more consumer friendly, easily available, and less expensive in developing countries than chemical preservatives.

Hurdle technology foods are in general less robust than traditional food products, which are often overprocessed and thus provide for a large margin of safety. Therefore, if modified hurdle technology foods are produced, the applied processes must be exactly defined and controlled. It is essential that soundly based challenge tests be applied before new hurdle technology foods are exploited in developing countries. Furthermore, strict guidelines must be employed in the processing of hurdle technology foods. The application of the Hazard Analysis Criti-

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cal Control Point (HACCP) concept still faces practical difficulties in developing countries, where many small producers prevail. However, guidelines based on Good Manufacturing Practice (GMP) are essential and often will be sufficient. The definition and control of such guidelines must be backed up by objective monitoring of the governing preservative factors, and even in small enterprises in developing countries, the most important of the preservative parameters such as time, temperature, pH, and a_w must be defined and should be quantitatively measured.

In recent years, impressive results have been achieved because of the introduction of hurdle technology for the microbial stabilization and sensory improvement of high-moisture foods in several countries of Latin America, especially fruits. But similar developments have started recently related to meat products of China and Argentina, as well as dairy products of India and miscellaneous foods of Africa. However, most of these developments are still in the pilot plant stage, but the industrial production of these modified or novel foods should be accomplished. If hurdle technology was employed on an industrial scale, seasonal surpluses of certain food commodities, such as fruits, could be accommodated, and therefore, post-harvest spoilage and waste of precious foods could be avoided, and fresh-like products would be available for consumption or further processing during the entire year.

Latin American researchers believe the application of intentional hurdle technology for food preservation might lead to an "explosion" of research and development of novel, minimally processed foods. On the other hand, if the time-honored preservation principles of traditional foods of developing countries become better understood, then food science in general would benefit, and traditional foods of developing countries might become a source of innovation for novel foods of industrialized countries.

Intentional hurdle technology might open up a new dimension of food preservation for both developing and industrialized countries. However, to achieve this goal more basic research is needed, which should focus on a better understanding of the homeostasis, stress reactions, and metabolic exhaustion of microorganisms, and especially on multitarget preservation of foods. The latter probably is the key for a breakthrough in the use of a truly intelligent application of hurdle technology. Analogous to the *combination therapy* in the medical field, which produced hopeful results in the use of chemotherapeutic drugs against tuberculosis, cancer, and recently HIV infections too, the *multitarget preservation* of foods[47] could be the most promising strategy for future food preservation in both industrialized and developing countries.

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15 Injured Bacteria

Bernard M. Mackey

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15.1 INTRODUCTION

The various methods used to preserve food are designed either to inactivate microbes of concern or to inhibit or prevent their growth. Organisms that survive such treatments may be injured so that they become more exacting in their growth requirements and fail to grow on the selective media normally used in their isolation. Under suitable conditions, injured cells can repair cellular damage and recover all their normal properties including virulence. An appreciation of the nature of sublethal injury and its repair is therefore important in detecting and enumerating foodborne microbes and in devising food preservation strategies.

Another class of microorganisms that have given rise to concern are those that enter the so-called viable but nonculturable (VBNC) state in response to starvation or other stresses. Such organisms lose their ability to grow on nutrient nonselective media that normally support their growth, but are believed to be viable because the cells remain physically intact and exhibit metabolic activity of various sorts. The suggestion that such organisms are capable of causing disease has serious implications in epidemiology and monitoring for food safety, but the physiological basis of the VBNC condition and its relationship to injured or dormant states is presently unclear.

15.2 DEFINITIONS OF VIABILITY, DEATH, AND SUBLETHAL INJURY

In microbiology, definitions of viability are usually based on the ability of organisms to multiply. Alternative definitions, based for example on the maintenance of homeostasis in single cells, are possible in principle but at present pose insuperable problems of measurement. Because viability is defined by the method of measurement, recovery conditions and the method for assessing cell multiplication must be specified. Colony formation or production of turbidity in broth are the usual criteria but there are numerous examples where cells may undergo limited multiplication to produce microcolonies on agar or final cell densities in liquid media that do not cause visible turbidity. The limitation of all definitions based on growth on or in laboratory media is that they exclude all those organisms that have not yet been cultured, estimated to comprise 90% or more of all microbes that inhabit the earth.[131] (Species that have been identified by gene probes but not yet cultured are often referred to as *hidden communities.*) Death of microbes may be defined as an irreversible loss of the ability to generate progeny. Sublethal injury can be reversed or circumvented whereas lethal injury cannot.

15.3 THE NATURE OF SUBLETHAL INJURY

15.3.1 Characteristics of Injured Bacteria

Many early workers described changes in the growth properties of bacteria following exposure to inimical conditions. An extended lag phase was observed in cells surviving heat or cold treatment[39,48,71] and more fastidious nutritional requirements for recovery were seen in heat-damaged or freeze-injured vegetative cells or spores.[40,70,124,167,175] The widespread use of selective enrichment media began in about 1920 but the limitations of these media in recovering stressed cells were not fully appreciated until quantitative studies revealed poor recovery of cells following freezing,[66] heating,[95] or drying.[127] Sublethal injury and its repair have been reviewed in numerous articles and book volumes.[5,16,30,75,77,120,168] Table 15-1 summarizes the characteristic features of injured vegetative cells and attempts to explain their altered growth properties in terms of damage to specific cell structures or functions.

15.3.2 Damage to the Gram-negative Outer Membrane

The outer membrane of Gram-negative bacteria protects the cell against lytic enzymes, bile salts, and certain antibiotics, but allows low molecular weight nutrients to diffuse into the periplasmic space where they may be taken up by transport systems present in the cytoplasmic membrane (Figure 15-1). If the outer membrane is damaged, cells become sensitized and unable to grow on selective media containing bile salts or triphenylmethane dyes (eg, MacConkey or Brilliant Green agars). Injury of this sort is typical of cells exposed to heat, cold shock, freezing, drying, aerosolization, and high pressure.

The phenotype of such injured cells resembles that of enteric organisms that have defects in the lipopolysaccharide (LPS) component of the outer membrane, arising from mutation or treatment with EDTA. Increased sensitivity to hydrophobic antibiotics in these cells is attributed to a conformational change resulting in exposure of hydrophobic regions on the outer membrane surface.[125] The corresponding structural basis of damage caused by physical treatments is not yet known. Loss of LPS has been seen in heat- or freeze-injured *Escherichia coli* and *Pseudomonas* cells,[72,158] and a conformational change in the outer membrane of freeze-injured *E. coli* was indicated by an altered pattern of adsorption of LPS-specific bacteriophages.[89,148] Increases in cell surface hydrophobicity and sensitivity to hydrophobic antibiotics were demonstrated in *E. coli* cells injured by heating, freezing, drying, or gamma-radiation.[103] However, changes in surface hydrophobicity alone would not be sufficient to account for the increase in sensitivity to vancomycin and lysozyme (which are large hydrophilic molecules), the leakage of periplasmic enzymes, or the increased permeability to dextrans of molecular mass 60,000–90,000.[32] Such gross changes in permeability suggest the creation, at least temporarily, of small holes or pores in the outer membrane.

15.3.3 Damage to the Gram-positive Cell Wall

Gram-positive organisms do not possess an outer membrane but some species have a surface protein layer that appears to have an analogous protective function. For example, in Gram-positive organisms that inhabit the gut, possession of a surface layer provides protection against bile salts and lytic enzymes. Freeze injury to *Lactobacillus bulgaricus* re-

Table 15-1 Sublethal Injury in Vegetative Cells

Symptom of Injury	*Suggested Cause of Injury*
Sensitivity to acid, alkali, osmotic stress; and some inorganic ions (eg, selenite, copper) Leakage of UV-absorbing material	Cytoplasmic membrane damage
Sensitivity to bile salts, triphenyl methane dyes, hydrophobic antibiotics, lytic enzymes Leakage of periplasmic enzymes	Damage to Gram-negative outer membrane or Gram-positive surface protein layer
Sensitivity to oxidative stress	Inactivation of catalase, superoxide dismutase Increased metabolic production of H_2O_2 or O_2^- Loss of cofactors for DNA repair enzymes Loss of intracellular reductants
Altered nutritional requirements for growth (real or apparent)	Enzyme inactivation (eg, peptidases) Membrane damage leading to sensitivity to Cu^{++} or growth stimulation or inhibition by amino acids Sensitivity to peroxide in rich media Metabolic imbalance on nutritional upshift
Altered metabolism	Enzyme inactivation Membrane damage leading to changes in enzyme/substrate accessibility
Increased frequency of mutation	Damage to DNA
Restricted temperature range for growth Increased sensitivity to secondary stress	Imbalance between repair and degradative processes (?)
Loss of virulence	(?)
Extended lag	Need to resynthesize damaged membranes, nucleic acids, ribosomes, etc

sulted in loss of surface protein and increased sensitivity to Tween 80.[197]

The cell wall of *Staphylococcus aureus* contains negatively charged teichoic acid polymers that bind magnesium. When heated in phosphate buffer, cells lost magnesium and D-alanine from wall teichoic acid.[74] In the absence of alanine, teichoic acid binds magnesium so strongly that it becomes unavailable for metabolic processes required for growth. If alanine is withheld from heat-injured cells, subsequent growth is very slow and occurs only after a long lag.

15.3.4 Damage to the Cytoplasmic Membrane

An intact cytoplasmic membrane is essential for the maintenance of homeostasis under fluctuating external conditions. Cells with damaged cytoplasmic membranes are inhibited by pH values or salt concentrations that would normally permit growth, and are hypersensitive to traces of copper in diluents or media. These symptoms of injury are associated with almost all forms of physical stress and could, in principle, be due to (1) gross loss of structural integrity or (2) loss of specific permease functions.

Gross damage is indicated by leakage of UV-absorbing material from the cell or increased access of molecules to the cytoplasmic compartment. Leaked material includes K^+, inorganic phosphate, phosphorylated sugars, sugars, fatty acids, esters, and amino compounds. An impression of the extent of injury is obtained from the size of molecule able to pass across the membrane. The cytoplasmic enzyme glucose-6-phosphate dehydrogenase was unable to escape from freeze-injured *E. coli*, whereas molecules of molecular mass 12 kD (ribonuclease) but not 16 kD (dextrans) were able to enter the cell from out-

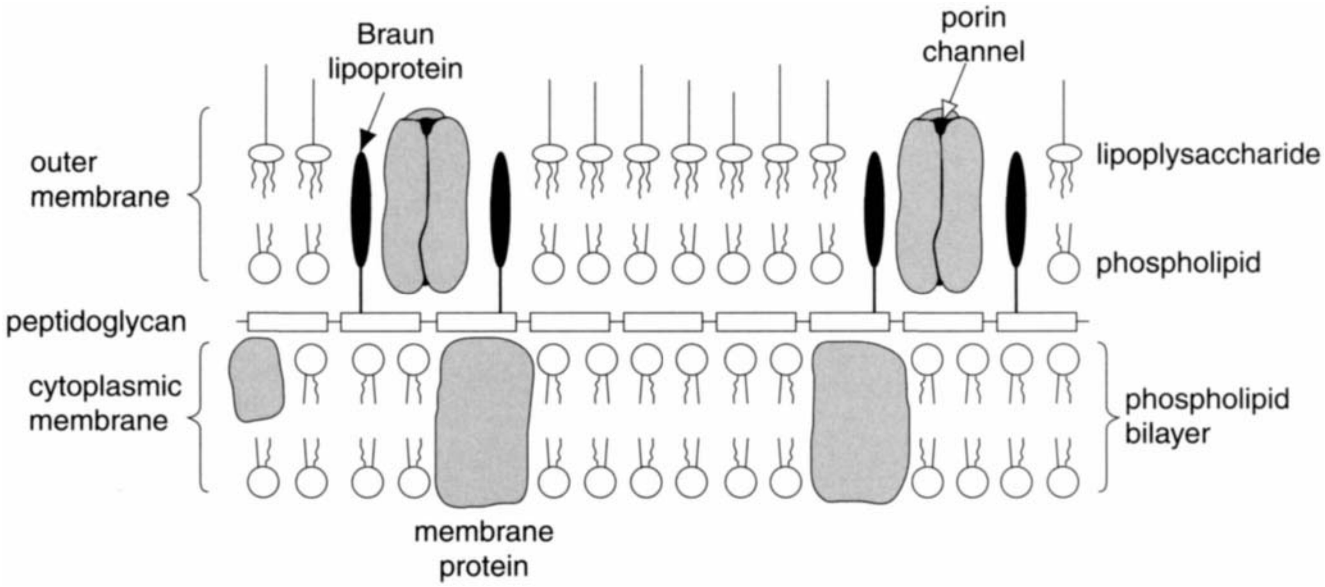

Figure 15-1 Schematic Representation of the Cell Envelope of an Enteric Gram-negative Bacterium.

side.[112,176] The amount and nature of material that leaks from damaged cells depends on the stress conditions. For example, cells of *Escherichia coli* frozen in water at freezing rates below 6°C/min lost no UV-absorbing material whereas above this rate, the release of UV-absorbing material was inversely related to survival. Leakage was not observed in acid-injured cells of *Salmonella bareilly* or *E. coli*.[22,143]

Gross structural damage in the form of loss of lipid and other membrane components during heating or freezing has been demonstrated in *E. coli* and *Staphylococcus aureus*.[78,170] By contrast, no lipid loss was detected in *Salmonella typhimurium* heated at 48°C, although cells became sensitive to eosin-methylene blue medium containing 2% NaCl.[180] During repair of heat injury, *de novo* synthesis of membrane lipid has been shown in *Salmonella typhimurium* and *Staphylococcus aureus*, and in both there was an increase in the proportion of unsaturated fatty acids in phospholipid synthesized during repair.[181]

Visual evidence for membrane damage in *E. coli* has been obtained by using light or electron microscopy. Cells exposed to mild heat released membrane vesicles into the medium, whereas cells exposed to hypotonic stress shed small membrane vesicles *into* the cytoplasm.[163,183] In pressure-treated *Listeria monocytogenes*, visible evidence of membrane perturbations took the form of large clear areas in the cytoplasm adjacent to membranes, suggesting that membrane invaginations had occurred under pressure.[109] In both bacteria and yeasts, rapid water movements in response to sudden changes in water potential cause loss of viability attributed to damage to the cytoplasmic membrane.[113,138]

Loss of homeostasis could also result from loss of specific membrane functions in an otherwise intact membrane. Alterations in the uptake or efflux of sugars and amino acids have been reported in several bacterial species and, in yeast, sublethal injury causes a lowering of the intracellular pH.[38,63,78,136] Partial inactivation of membrane-bound ATPases occurred during pressure treatment of *Lactobacillus plantarum*,[169] and a key role for this enzyme in thermotolerance of the yeast *Saccharomyces cerevisiae* has been postulated.[38]

The cytoplasmic membrane is clearly an important target for damage by inimical treatments but it is not yet clear what distinguishes lethal from sublethal damage. Temporary loss of permeability control is not necessarily fatal, as seen in electroporated cells that are permeable to large DNA molecules but can recover full viability if incubated in a favorable medium, as can those damaged by bacteriocins.[188] Membrane damage presumably becomes lethal if (1) the damage is of a nature that precludes resealing, or (2) irreversible effects occur as a secondary consequence of loss of permeability control, energy conservation, or other membrane functions. Loss of magnesium can lead to the destabilization of ribosomes and failure of DNA repair processes, while loss of other cofactors will disrupt normal metabolic control processes. If this perturbed condition persists for long enough and the supply of energy is curtailed, the degenerative changes become impossible to reverse and cell death ensues. Irreversible effects are especially likely when the external environment differs markedly from that of the cell or when inhibitors or other deleterious agents are present in the medium. In any population of injured cells, there is a spectrum of sensitivity to salt, pH, or other agents, implying varying degrees of membrane injury, but the physical basis of this is completely unknown.

15.3.5 Damage to DNA

Damage to DNA is the primary cause of cell death in microbes exposed to ultraviolet or ionizing radiation. Ultraviolet light, particularly at 250 to 260 nm, is absorbed by nucleic acids, giving rise to cyclobutane pyrimidine dimers that distort the DNA helix. Ionizing radiation causes a wide range of damage products in DNA, but the most important biologically are single- or double-strand breaks. *Escherichia coli* can remove up to 850 pyrimidine dimers and can repair up to 30 single-strand breaks per genome in the exponential phase.[119] *Deinococcus radiodurans* is exceptionally resistant to UV and ionizing radiation and can deal with around 12,000 pyrimidine dimers and up to 3,000 single-strand or 240 double-strand breaks. DNA damage is therefore sublethal up to the point where the repair systems become saturated. The ability of repair systems to cope with the damage is influenced by post-injury holding conditions. Survival is enhanced if irradiated cells are incubated in buffer before plating out because this allows more time for repair before chromosome replication is attempted. As a general rule, plating media that support rapid growth result in the lowest recoveries of irradiated cells. The optimum temperature for recovery of irradiated bacteria is strain dependent but is often lower than the optimum for growth. Survival of gamma-irradiated cells of *Escherichia coli* B/r was thus maximal around 18°C, compared with a growth optimum of 37°C.[88]

DNA damage has also been reported in heated, freeze-thawed, dried, and acid-treated cells, measured directly as single- or double-strand breaks, or inferred from an increase in the mutation frequency, or greater sensitivity to stress of mutant strains defective in DNA repair processes.[8,28,106,135] DNA damage can occur directly as a result of acid-catalyzed deamination or depurination,[99] or indirectly, caused by nuclease action. Mutants of *E. coli* lacking the *xthA* encoded apurinic endonuclease were more resistant to heat and sustained fewer strand breaks in their DNA than the parental strain.[64]

The significance of DNA damage in death caused by heating, freezing, drying, or acid is not clear. The number of strand breaks reported in freeze-injured *E. coli* at 50% survival was only about three,[3] a number easily repaired in irradiated cells. If repair systems were also inactivated by the stress treatment, a low number of lesions normally regarded as sublethal might become lethal. In cold-shocked cells, death was attributed to loss of magnesium that is required for the action of DNA-ligase, an enzyme involved in DNA synthesis and repair.[160]

With ionizing radiation damage it has been possible to relate recovery of viability to repair of DNA by performing fractionated dose experiments in which recovery after a set dose of radiation is increased if an interval for DNA repair is allowed during the administration of the dose.[54] There are no corresponding data relating DNA repair to recovery of cells damaged by other agents, but the release of heat-shock proteins from the nucleoid of heat-injured *E. coli* during incubation in broth at 37°C was correlated with recovery of ability to grow on agar plates.[135]

15.3.6 Damage to Ribosomes and RNA

Loss of ribosomes occurs in heat-injured and starved cells especially under conditions that lead to depletion of intracellular magnesium. When cells of *Salmonella typhimurium, Staphylococcus aureus, Pseudomonas fluorescens,* or *Agrobacterium tumefaciens* are heated in phosphate buffer at relatively mild temperatures (48°–50°C) selective destruction of the 30S ribosomal subunit and 16S RNA is observed.[76,181] This effect is attributed to destabilization of ribosome structure by loss of magnesium followed by nuclease attack. Destruction of the 30S subunit is prevented when magnesium is added to the buffer in which cells are heated. Loss of the 30S subunit is not necessarily lethal under these mild heating conditions and resynthesis of 16S RNA and ribosomal proteins can occur when cells are incubated in growth medium at 37°C. Death of exponential phase cells of *Salmonella typhimurium* at 52°C was accompanied by extensive degradation of 16S RNA. Starved cells or those grown in the presence of 100 mM $MgCl_2$ were not killed under the same heating conditions and retained 16S RNA, hence it was concluded that degradation of ribosomal RNA is a direct cause of cell death. Studies of cells heated at rising temperatures in the differential scanning calorimeter showed that loss of viability in *E. coli* and *Listeria monocytogenes* was associated with irreversible destruction of the 30S subunit at higher temperatures.[4,110] Loss of the 30S ribosomal subunit can thus be lethal or sublethal depending on heating and recovery conditions. This form of injury is associated with an extended lag time during which RNA and protein are resynthesized.

15.3.7 Altered Nutritional Requirements for Growth

Injured cells usually recover better on rich than minimal media. The added nutrients may aid recovery simply by removing some of the metabolic burden from injured cells, but in freeze-injured *Escherichia coli* and *Streptococcus lactis*, specific requirements for amino acids or peptides were identified.[106,133] In some strains of *E. coli*, failure to grow on minimal medium was not a nutritional effect. Cells with damaged membranes were sensitive to traces of copper ions present in water used to make the media, and in rich media, copper ions were chelated by amino acids and hence detoxified. A quite different example of an apparently altered nutritional requirement after exposure to stress was seen in *E. coli* cells treated with N-ethylmaleimide.[182] Following exposure to this compound, cells lost the ability to grow on minimal agar but could do so after supplementation with peptone. This was not a nutritional effect because different peptones, each of which was sufficient to support good growth, differed in their ability to prevent loss of colony-forming ability on plates. It was suggested that depletion of intracellular glutathione caused cells to be sensitive to reactive oxygen intermediates, and peptides served a protective function in place of glutathione.

Most bacteria in marine samples are unable to grow on rich nutrient media, but may develop the ability to do so after prolonged storage in the stationary phase or on subculture to progressively higher nutrient concentrations.[53,162] The oligotrophic phenotype appears to be associated with adaptation to low nutrient conditions and possession of very high affinity uptake systems, but the physiology is poorly understood. The phenomenon of substrate-accelerated death in starved copiotrophic bacteria is well documented[43,142] but the significance of *metabolic imbalance* in recovery of stressed cells generally, requires clarification.

15.3.8 Sensitivity to Oxidative Stress

The sensitivity of injured cells to oxidative stress is of major importance in their detection and survival but is often little appreciated. Exposing complex growth media to light in the presence of air results in the photochemical production of peroxide and superoxide whose presence is lethal to injured cells. The addition of catalase or pyruvate removes peroxide and improves recovery of injured Gram-positive and Gram-negative bacteria.[115] Incubation of heat-injured cells under strictly anaerobic conditions also dramatically improved recoveries of *L. monocytogenes, E. coli* O157, *Staphylococcus aureus*, and *Salmonella enteritidis*.[55,92]

The phenomenon of *minimal medium recovery* may also be interpreted as an effect of oxidative stress. Under some conditions injured cells recover better on minimal salts media than on a rich media such as tryptone soya agar. This occurs in cold-shocked or heat-injured Gram-negative bacteria, but is usually seen only when cells are (1) grown in a minimal medium and (2) stressed during the exponential phase of growth. Cells exposed to the rich medium apparently die as result of oxidative damage to DNA caused by traces of peroxide present in the medium.[57,107]

The increased sensitivity to oxidative stress from exogenous sources could be caused by increased susceptibility of target sites to oxidative attack or decreased efficiency of protective mechanisms. Inactivation of catalase and superoxide dismutase has been recorded in heated cells of *Staphylococcus aureus* and *E. coli*, but the relationship between loss of enzyme activity and sensitivity to oxidative stress and/or sensitivity to heat is not simple. Heating *S. aureus* at 52°C for 45 minutes reduced viable counts by 90% but reduced superoxide dismutase activity by only 10%.[29] Catalase activity decreased only slightly during heating at 52°C, but a much greater decrease occurred during the first two to four hours of repair in tryptone soya broth.[6] In *E. coli*, increased sensitivity to micromolar concentrations of peroxide in heat-injured cells was not correlated with loss of total catalase activity but matched the decline in activity of the inducible hydroperoxidase I.[108] Relatively mild dilution shock may also render cells susceptible to oxidative stress,[107] suggesting that other factors may also be involved, eg, loss of cofactors needed for DNA repair.[159]

Strains of *Escherichia coli* with null mutations in *sodAsodB*, the genes encoding superoxide dismutase, are sensitive to heating aerobically at 48°C, whereas the same stress applied to the parent strain had negligible effect.[15] This effect was not seen at 55°C, suggesting that at higher temperatures direct thermolability of other cell components overrides the effect of superoxide radicals. The metabolic flux of superoxide can vary widely depending on the metabolic substrates used as a source of electrons and the pathway of electron transport.[80] It is possible that metabolic imbalance or disorganization of the electron transport chain in damaged membranes could lead to increased production of superoxide, with lethal consequences. This has not been tested directly, but preliminary evidence has been obtained, using chemiluminescent probes of a burst of free radical production in *E. coli* following heat, osmotic, and ethanol shock.[2]

15.3.9 Effects of Temperature on Recovery

The optimum temperature for recovery of injured cells is often below the optimum for growth.[17,111] Figure 15-2 shows that incubation temperatures between 5° and 40°C had no effect on the plating efficiency of uninjured cells, whereas injured cell exhibited an optimum for recovery between 20° and 25°C. Incubating plates outside this range could reduce viable counts of injured cells by up to 500-fold. The reasons for this are not clear and are likely to involve a balance between the relative rates of repair processes and rates of degenerative changes leading to death in injured cells.

15.3.10 Metabolic Requirements for Repair of Injury

The repair of sublethal injury can be followed by using the differential plating method. After exposure to an injurious treatment, cells are incubated in broth and plated at intervals on an optimum medium to determine the total number of viable cells, and on a restrictive medium (eg, one containing bile salts or sodium chloride) that allows growth only of uninjured cells. The difference between the two counts gives the proportion of injured cells, and completion of repair is indicated when cells have regained ability to grow on the restrictive medium (Figure 15-3).

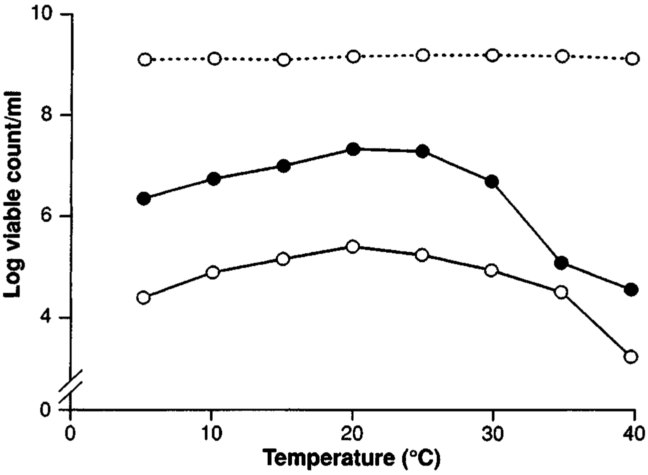

Figure 15-2 Effect of Temperature on Recovery of Heat-Injured *Listeria monocytogenes*. Viable counts of unheated cells (---O---); cells heated at 60°C for 10 min (●) or 20 min (O). *Source:* Reprinted with permission from B.M. Mackey et al., Recovery of Heat-Injured *Listeria Monocytogenes*, *International Journal of Food Microbiology*, Vol. 22, pp. 227–237, © 1994, Elsevier Applied Science.

The metabolic requirements for repair of injury have been studied by measuring the incorporation of radiolabeled precursors into cell components in the lag preceding multiplication or by examining the effect of metabolic inhibitors on the repair process. Tabulated summaries of the biosynthetic requirements for repair were provided by Tomlins and Ordal,[181] Mackey,[106] and Mossel and Corry,[120] and examples for *Salmonella* species are shown in Table 15-2. It is evident that biosynthetic requirements for repair vary between different stress treatments and can also vary for the same type of stress under different conditions (eg, chilling in water versus broth).

15.4 VIABLE BUT NONCULTURABLE CELLS

15.4.1 Characteristics of Viable but Nonculturable Cells

Recent studies, in which ribosomal RNA gene sequences are amplified by PCR directly from natural environments without first culturing the organisms, have amply demonstrated that a large proportion of bacteria present in the oceans, hot springs, and other ecological niches do not grow on standard laboratory media.[131] It is supposed that these so-far uncultured cells require special factors to permit growth, or are inhibited by the media used in attempts to isolate them. Earlier studies showed that the number of cells that could be cultured from marine or soil samples represented only a small fraction of the total that could be detected microscopically. It was suggested that bacteria in these environments spend much of their existence in a state of metabolic stasis or dormancy interspersed by short periods of growth.[171] It was further supposed that the dormant fraction was identical to the nonculturable fraction, although some recent studies have suggested that up to 50% of nonculturable cells in marine environments are not dormant and can be shown to be capable of multiplication in seawater but not on rich media.[31,161] The literature on dormancy of nonsporulating bacteria has been reviewed by Kaprelyants et al.[86] and Roszak and Colwell.[155]

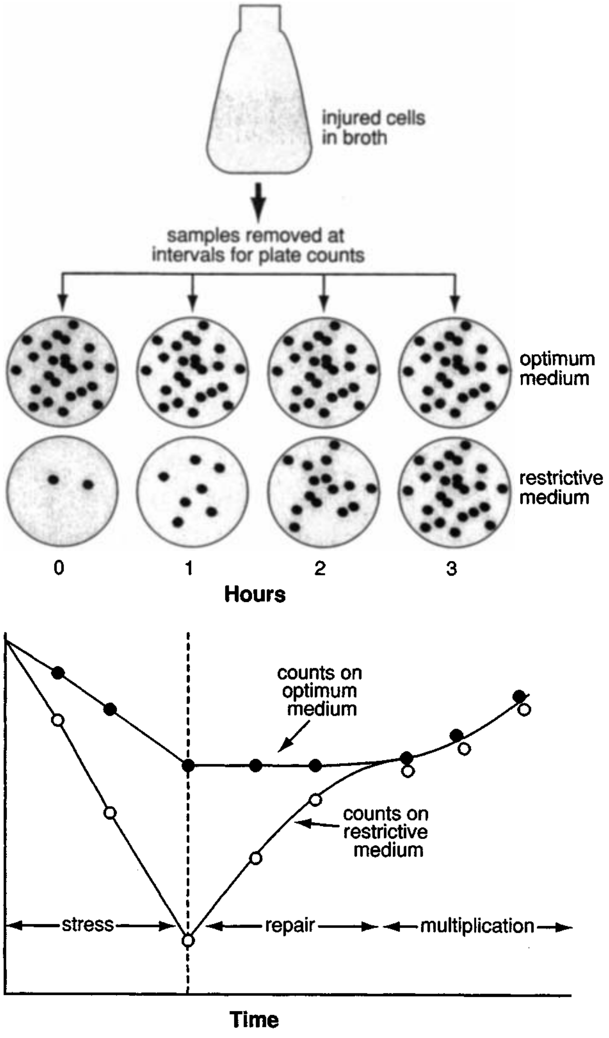

Figure 15-3 Method of Following Repair of Injury Using the Differential Plating Method.

In the present context, the term viable but nonculturable (VBNC) is applied in a more restricted sense to describe what is believed to be a temporary loss of culturability in bacteria that can normally be cultivated with ease. The term was first used by Colwell's group in 1982 to describe a phenomenon in *Escherichia coli* and *Vibrio cholerae* in which organisms that were incubated in salt water microcosms lost their ability to grow on nutrient agar plates but remained metabolically active over long periods.[198] There is no generally agreed definition of the VBNC state and in common usage it is rather loosely applied to (1) organisms that fail to grow on nutrient nonselective media that normally support their growth but which retain cellular metabolic activity measured in one of several different tests (see Section 15.4.2) and (2) organisms that become nonculturable but can regain the capacity to multiply on their normal growth media following a suitable resuscitation treatment. It should be noted that dormancy is defined simply as a state of rest or metabolic quiescence in which the phenotypic development of an organism is interrupted.[86,177] Therefore, dormancy is not necessarily associated with nonculturability and vice versa. Nevertheless, entry into a VBNC state in vegetative cells is often viewed as being analogous to spore formation in which cells become dormant as a means of surviving adverse conditions.

Barer[11,12] pointed out that since viability can only be definitely established by showing that cells can multiply, the term viable but nonculturable is in fact an oxymoron. An alternative terminology is suggested below (see Section 15.4.4) but, in the absence of a more precise agreed alternative, the term VBNC,

Table 15-2 Metabolic Requirements for Repair of Sublethal Injury in *Salmonella* Species

Organism	*Treatment*	*Protein Synthesis*	*RNA Synthesis*	*DNA Repair*	*ATP Synthesis*	*Cell Wall Synthesis*	*Reference*
S. typhimurium	Heat	+*	+	+	+	0	181
S. bareilly	Acid	+	+	0	+	0	22
S. heidelberg	Cold shock	0	+	ND	0	0	178
S. heidelberg	Chilled storage						179
	(a) water	0	0	ND	+	+	
	(b) broth	0	0	ND	0	0	
S. anatum	Freeze thaw	0	0	ND	+	0	150
S. anatum	Freeze dry	0	0	ND	+	0	149

*+, synthesis required; 0, synthesis not required; ND, not determined.

as commonly used in the literature, will be retained in this discussion for convenience.

The VBNC state has been reported in many genera, mainly of Gram-negative organisms but including the Gram-positive *Micrococcus luteus, Listeria monocytogenes*, and *Enterococcus faecalis*. The most studied organisms have been *Escherichia coli, Vibrio cholerae, Vibrio vulnificus, Legionella pneumophila, Campylobacter jejuni, Salmonella enteritidis*, and *Micrococcus luteus*.[12,116] In most cases, the inducing stress is starvation in fresh or seawater, but VBNC cells have also been reported in response to biocide treatment, osmotic stress, exposure to sunlight, oxidative stress, aerosolization, and survival in soil. The nature of the VBNC state will be discussed below (see Section 15.4.3), but it is first worth summarizing the types of evidence on which the existence of the state is based.

15.4.2 Evidence for the VBNC State in Organisms That Can Normally Be Cultured

Epidemiological Evidence

Several ecological studies suggest that viable cells of pathogenic bacteria may be more numerous in aquatic environments than are detected by culture methods. In areas of the world where cholera is endemic, the isolation of *Vibrio cholerae* from water samples shows distinct biyearly peaks. At other times of the year, *V. cholerae* cannot be detected by culture methods but cases of cholera still occur. In contrast to the fluctuating pattern obtained by conventional culture methods, when water samples are examined using fluorescent antibody techniques, *V. cholerae* can be detected throughout the year. A similar seasonality is observed with *V. vulnificus*, which apparently disappears from coastal waters of the Northeast United States between October and June when the temperature drops to below 10°C. Estimates of the number of *V. fischeri* cells in marine samples based on plate counts were about 100 times lower than those based on quantitative DNA hybridization or ability to colonize the light organ of baby squid.[97] Plate counts also apparently underestimate numbers of *Legionella pneumophila* in tap water because fluorescent antibody tests indicated the presence of the organism in winter when none were detected by culture methods.[35] The evidence from epidemiology is of course circumstantial, and the detection of cells by nucleic acid or antibody-based methods does not prove that viable cells exist where none can be detected by culture methods. Nonetheless, these studies would be consistent with the existence of a VBNC state in natural environments.

Direct Indicators of Viability

Detection of metabolic activity at the single cell level and/or possession of an intact cell membrane is often taken as presumptive evidence of viability in nonculturable cells. The techniques listed in Table 15-3 have been applied to populations of organisms or, using fluorescence microscopy or flow cytometry, to single cells.[12,85,141,155] These techniques have shown that nonculturable cells can exhibit many properties associated with living cells including electron transport activity, maintenance of a membrane potential, induced enzyme synthesis, and ability to receive and express plasmids by conjugation.[6,128,140]

The direct viable count (DVC) method of Kogure[93] is one of the most common criteria of viability in studies of the VBNC state. In this test, cells that can elongate when incubated in medium containing yeast extract and an inhibitor of DNA synthesis are considered to be alive. Roszak et al.[156] found that plate counts of *Salmonella enteritidis* in river water declined to zero within 3 days whereas the ability of cells to respond in the Kogure DVC persisted for up to 60 days. It was concluded that cells had entered a dormant state. The persistence of a DVC response in nonculturable cells is very variable and, in some cases, the DVC can decline as rapidly as culturability.[24]

Organisms that have been killed by radiation retain extensive metabolic capacity including the ability to synthesize protein and to support propagation of bacteriophage. Similarly, *minicells* that lack DNA and are incapable of replication would give a positive response in many of the direct viability tests. The presence or absence of detectable metabolic activity is therefore not an unequivocal indicator of viability.

Resuscitation In Vitro

The most convincing verification of the VBNC state is to demonstrate loss and recovery of culturability under controlled conditions in the laboratory. The best characterized resuscitation system for Gram-negative bacteria has been that employing the marine pathogen *Vibrio vulnificus*.[126] When grown in artificial seawater and then chilled from a temperature of 22°C to around 10°C, the cells change in morphology from rod to coccoid shape and rapidly become nonculturable, but direct viable counts remain high. When the nonculturable population is warmed to 20°C, there is a rapid increase in plate counts accompanied by a reversal of the morphological change.

Table 15-3 Some Indicators of Single Cell Metabolic Activity

Function Measured	*Example of Method*
Membrane potential	Active uptake of Rhodamine 123 Exclusion of bis-oxonol
Membrane integrity	Exclusion of propidium iodide
Electron transport activity/generation of reducing power	Reduction of tetrazolium salts
DNA synthesis	Incorporation of radiolabeled thymidine
Protein synthesis	Incorporation of radiolabeled amino acid
Enzyme activity	Hydrolysis of fluorescein diacetate or derivatives by nonspecific esterases Fluorogenic ß-galactosidase assay
Intracellular pH	pH-dependent fluorescence spectrum of carboxyfluorescein succinimidyl ester
Coordinated macromolecular synthesis	Cell elongation in the presence of DNA gyrase inhibitors Induced enzyme synthesis

This process may represent resuscitation of VBNC cells to full viability, but an alternative explanation is that the increase in viable number is caused by multiplication of residual culturable cells. In some experiments, resuscitation was dependent on culture volume (and therefore the probability of there being a culturable cell present), and seawater was shown to support cell multiplication.[189,191] Similar conclusions were drawn from experiments with the eel pathogen *V. vulnificus* biotype 2[21] and with *V. cholerae*[146] but, in later experiments, resuscitation of *V. vulnificus* present in artificial seawater was possible from samples containing less than 0.0001 cfu.[192] The probability that normally culturable cells were responsible for the increase in viable count was therefore extremely low in this instance.

Much of the uncertainty of resuscitation experiments could be removed by appropriate experimental design using a most probable number (MPN) technique (Figure 15-4). Suspensions of bacteria believed to contain VBNC cells are serially diluted in a liquid medium before being incubated under conditions that will allow resuscitation to occur. Cells that can initially grow on plates are diluted out by this procedure so any growth in tubes at greater dilutions must come from resuscitation of VBNC cells. By performing serial dilutions in replicate (eg, five replicates), the MPN can be calculated and compared with the plate count to give the proportion of VBNC cells in the population.

The Gram-positive organism *Micrococcus luteus* becomes dormant and also nonculturable when maintained for several months in a spent culture medium but can be resuscitated by incubation in fresh medium containing filter-sterilized culture fluid obtained from a culture in late exponential phase. The number of dormant viable cells, estimated by MPN counts in medium containing culture filtrate, was shown to be about 10,000 times greater than the number of cells able to grow on plates of normal growth medium.[87,186] However, similar MPN experiments with *V. vulnificus* employing a range of nutrient and dilute media and spent broth were unsuccessful at resuscitating cells from a nonculturable state.[189] Similarly, the MPN method demonstrated that the apparent resuscitation of *V. parahaemolyticus* cells after a temperature upshift represented growth of a few surviving cells.[81] When spent broth cultures of *C. jejuni* consisting predominantly of coccoid cells are sparged with a microaerophilic gas mixture there is a rapid increase in viable numbers accompanied by a change from a predominantly coccoid to vibrioid morphology. Application of the MPN approach showed that this could be largely explained by multiplication of residual culturable cells.[26] However, plate counts of aged cultures under supposedly optimum microaerophilic conditions underestimated the viable count by up to 20-fold, indicating that a fraction of survi-

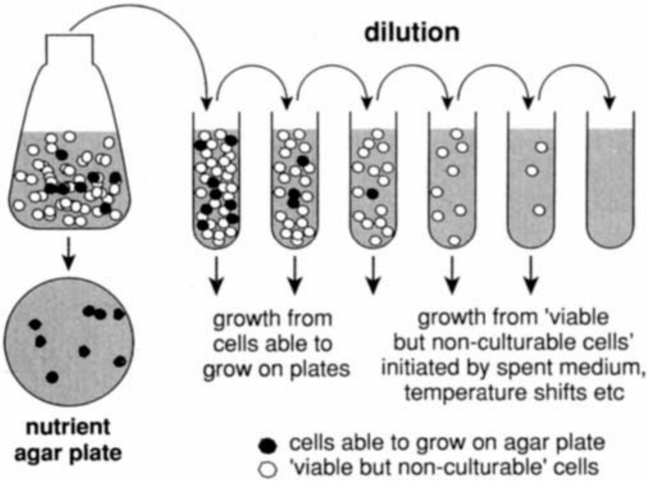
dilution
nutrient agar plate
growth from cells able to grow on plates
growth from 'viable but non-culturable cells' initiated by spent medium, temperature shifts etc
● cells able to grow on agar plate
○ 'viable but non-culturable' cells

Figure 15-4 Use of the MPN Approach To Distinguish Resuscitation from Multiplication.

vors were in an injured or latent state, though the numbers of such cells were much less than in starved *M. luteus*.[26]

Recovery of *Legionella pneumophila* from water samples that initially contained no detectable culturable cells was achieved by a mild heat-shock treatment.[35] In a different system, cells of *L. pneumophila* that had been nonculturable for 55 days in tap water did not respond in the Kogure DVC but were recovered by co-culture with *Acanthamoeba castellani*.[172] In this study, a heat shock had a detrimental effect on reactivation. Several protozoan species support the growth of *L. pneumophila,* and it will be interesting to discover the factors that promote recovery of starved cells. However, as with other resuscitation studies, it will be necessary to use the MPN approach to determine what fraction of remaining cells is actually capable of being resuscitated.

Pathogenicity of VBNC Cells

Colwell et al.[36] first reported that cells of *V. cholerae* rendered nonculturable by incubation in estuarine water microcosms retained virulence when tested in rabbit ligated ileal loop assays, and later reported that the VBNC form of a vaccine strain could revert to culturability when fed to human volunteers.[37] Evidence for virulence of VBNC cells has been produced for several other species but in some cases the evidence is contradictory (Table 15-4). For example, Jones et al.[83] were able to recover culturable cells of *C. jejuni* from the VBNC state following feeding to suckling mice whereas Medema et al.[117] and Beumer et al.[18] were unable to demonstrate reversion, colonization, or infection in a variety of different systems and Fearnley et al.[50,51] found that the ability to colonize chicks was lost before cells became nonculturable (Table 15-4). The reasons for these discrepancies are not known but may be related to the method of producing VBNC cells and the length of time they have been in the VBNC state. Feeding and infectivity studies have been criticized for the same reasons as *in vitro* resuscitation studies on the grounds that infection could have been due to culturable cells that were undetected because of sampling errors in the viable count procedures. This argument requires either that appreciable numbers of cells were missed by the viable count method or that the number of surviving cells in the inoculum, and hence the infective dose, is very low. These objections might be overcome by more quantitative

Table 15-4 Infectivity or Virulence of VBNC Cells

Organism	*Inducing Treatment*	*Assay System*	*Result*	*Reference*
Vibrio vulnificus	Temperature downshift	Injection into mice	+	129
Vibrio cholerae	Estuarine water	Rabbit ligated ileal loop	+	36
Vibrio cholerae (vaccine strain)	Phosphate buffered saline	Human volunteers	+	37
Legionella pneumophila	Tap water	Injection into embryonated eggs	+	79
Campylobacter jejuni	Pond water	Suckling mice	+	83
Campylobacter jejuni	Phosphate buffered saline	Chick colonization	+	174
Campylobacter jejuni	Freeze thaw	Rat ileal loop	+	159
Campylobacter jejuni	Reservoir water	Inoculation into embryonated eggs	–	117
		Chick colonization	–	
Campylobacter jejuni	Distilled water or freeze thaw	Chick colonization	–	50, 51
Campylobacter jejuni	0.85% saline	Feeding to: mice, rabbits, human volunteers	–	18
Helicobacter pylori	20 days in broth	Intragastric inoculation into mice	+	34
Aeromonas salmonicida	Seawater	Infection of fish	–	154
Shigella dysenteriae	Distilled water	Detection of Shiga toxin	+	144
		Adherence to Henle cells	+	
		Invasion of Henle cells	–	
Enterotoxigenic *Escherichia coli*	Seawater/sunlight	Rabbit intestinal loop	+	139
		ELISA for enterotoxin	+	

data although this is difficult because of the inherent variability of infective doses. Interpretation of results from model systems is also complicated by the multifactorial nature of bacterial virulence and, in some trials, VBNC cells responded in some but not all tests of virulence (Table 15-4). It would be helpful in these cases to compare the responses of VBNC cells with those of similar numbers of unequivocally dead cells (eg, killed by gamma-irradiation), but this is seldom done. Thus, although several studies have concluded that VBNC cells remain virulent and have taken care to avoid spurious results from viable cells being present, this conclusion remains controversial. The implications for public health of VBNC cells remaining infective are serious but, because of the conflicting experimental results, the pathogenicity of VBNC cells cannot yet be regarded as firmly established and the relevance to human infection of the VBNC state remains uncertain.

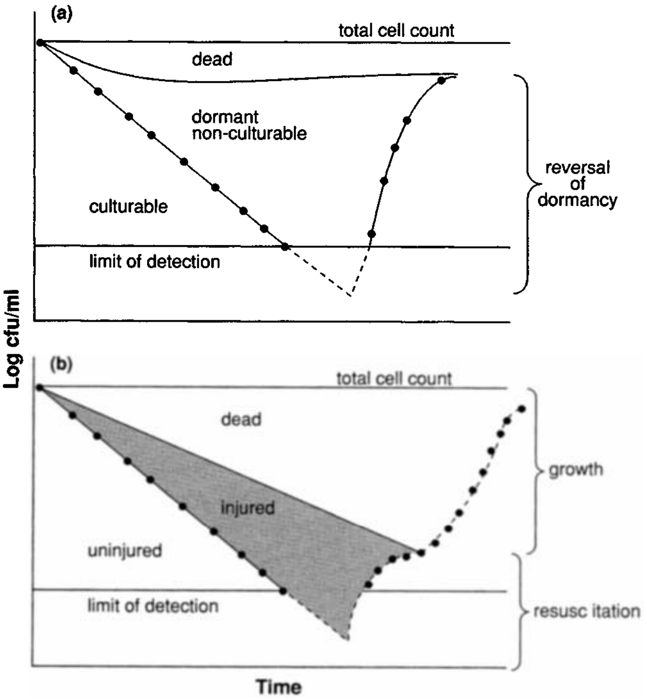

Figure 15-5 Two Interpretations of the Increase in Viable Numbers Seen during Resuscitation of VBNC Cells. **A**, The increase in viable count is due to a reversal of dormancy in a large proportion of the total cell population. **B**, The increase in viable count is due to repair of injury in a relatively small fraction of the population followed by multiplication. At the start of resuscitation, the number of uninjured cells is below the detection limit.

15.4.3 The Nature of the VBNC State

The VBNC state is commonly interpreted as a survival mechanism wherein cells become metabolically quiescent or dormant in response to adverse conditions. This is seen as being analogous to spore formation and is presumed to be a genetically programmed process. According to the dormancy hypothesis, cells can persist for long periods in the VBNC state, and the large increase in viable numbers that occurs during resuscitation is mainly or entirely due to the reversal of dormancy. This is shown schematically in Figure 15-5A. In *Micrococcus luteus*, a 16 to 17 kD_a protein has been identified in spent medium that is needed to reverse the dormant state.[123] Dormant bacterial endospores also require specific signals (germinants) to become active but, in this case, the signal molecules originate from the environment rather than other organisms. "Wake up" factors have not yet been identified for other VBNC cells although growth of the eukaryotic microbes *Tetrahymena* from very small inocula was specifically stimulated by 8-bromo cyclic GMP, a signal molecule present in spent medium.[145] The lag phase of starved ammonia oxidizing bacteria following resupply of ammonia was dramatically reduced by a homoserine lactone.[13]

There are several complicating factors in applying the dormancy hypothesis to all VBNC conditions: (1) one class of VBNC cells is in fact defined by its ability to display appreciable metabolic activity and thus cannot also be considered dormant; (2) the VBNC state may arise in response to a sudden stress such as aerosolization[69] that would preclude any cellular differentiation process; and (3) in at least two published papers, the nonculturable phenotype was observed in suspensions that were multiplying at the time of sampling, as judged from an increasing total count or DVC.[122,132]

An alternative explanation is that stressed cells are nonculturable because they are injured or otherwise in a physiological state that sensitizes cells to dilution protocols or components of recovery media, or which results in metabolic imbalance and death when cells are placed on nutrient agar plates. According to this view there could be many different causes of temporary nonculturability. Weichart and Kjelleberg[189] found that chilled cells of *V. vulnificus* recovered better on soft agar (0.2% to 0.8%) plates than on ordinary agar and Whitesides and Oliver[192] showed that Heart Infusion broth inhibited resuscitation of *V. vulnificus* (although it was not lethal during a 12-hour period of exposure). Mukamolova et al.[122] concluded that the filtered supernatant from a growing culture of *M. luteus* stimulated resuscitation of starved cells by overcoming the activity of an antibacterial factor, produced during resuscitation, that caused self-poisoning. Temporary nonculturability was induced in exponential phase *E. coli* as a result of osmotic stress but could be reversed by betain[157]; and in *L. monocytogenes*, a similar temporary loss of colony-forming ability in osmotically stressed cells was correlated with membrane damage detected by ethidium bromide staining.[153] The role of oxidative stress in the recovery of stressed cells was emphasized above and could also be important in the VBNC phenomenon.

The shape of the resuscitation curves in published data is sometimes characterized by an initial rapid increase in viable numbers followed by a plateau then a second increase.[130] This would be consistent with an initial phase of resuscitation followed by a lag preceding multiplication (Figure 15-5B). The period during which it is possible to resuscitate cells beyond the point at which plate counts become zero, would thus depend on the ratio of injured to uninjured cells and the rates of decline of both.

A third possibility is that, in some cases at least, the putative VBNC cells are actually dead (incapable of multiplication under any conditions) but retain metabolic capacity for a period that depends on the relative rates of decay of a function essential for multiplication and of other cell functions. Weichart et al.[190] postulated that there are two phases in formation of nonculturable cells of *V. vulnificus*: the first involves loss of culturability with maintenance of cellular integrity (and possibly viability) while the second is typified by gradual degradation and loss of nucleic acids. Boucher et al.[25] showed that nonculturable cells of *Campylobacter jejuni* could reduce tetrazolium salts but that the ability to do so declined with time and concluded this was a gradual degenerative process leading to cell lysis. In *C. jejuni* conversion to the coccoid form was not prevented by inhibitors of protein or RNA synthesis or by irradiating the cells, demonstrating that the process was not an active one.[68] Coccoid cell formation in *Helicobacter pylori* was accompanied by a reduction in the amount and integrity of RNA and DNA and was also not prevented by inhibitors of protein or RNA synthesis. Nonculturability occurred while 50% of cells were still in the bacillary form, and it was concluded that the coccoid form of *H. pylori* is a morphological manifestation of cell death.[94]

There is an increasing tendency in the literature to label all physically intact cells that cannot be recovered, but which exhibit metabolic activity or can be detected by gene probe methods, as VBNC. It is now very well established that such cells retain many of the properties of living cells for a long time after culturability has been lost, but the evidence that they also remain capable of reproduction is often weak. At the time of writing, resuscitation of VBNC cells has been unequivocally demonstrated in relatively few cases, and in only one instance (*M. luteus*) has it been shown that a large proportion of the total cell population is capable of being resuscitated. In view of the large body of circumstantial evidence that a VBNC state may exist in many organisms, and the wide implications that this would have for monitoring and safety, there is an urgent need for better designed resuscitation experiments to establish the true extent and significance of the phenomenon. There may be many quite separate reasons for temporary nonculturability, and it is essential to understand the physiological mechanisms involved and the properties, including resistance, of VBNC cells.

15.4.4 Terminology

If one accepts that the definition of a viable bacterial cell is one that is capable of multiplying under suitable conditions, then there is no justification for labeling cells as viable unless there is good evidence that they can indeed multiply. A term such as (metabolically) active but nonculturable (ABNC) would be a nonprejudicial descriptor for organisms currently described as VBNC on the basis of their response in the Kogure DVC, ability to reduce tetrazolium dyes or other similar tests. In cases where resuscitation, and hence viability, has been demonstrated, but the cause of the temporary inability to grow on agar plates is not known, cells could be described as *fastidious* (or to have entered a fastidious state), a generic term describing cells that require special conditions to initiate growth, whether as a result of dormancy, metabolic imbalance, injury, or any other reason. An active but nonculturable (ABNC) cell could thus be dead, or alive but fastidious. Once the basis of nonculturability is discovered, more specific descriptors of the cell's physiological state could be used.

15.5 SPORE INJURY

15.5.1 The Occurrence of Spore Injury

The pioneering work of Bigelow and Esty in 1920[19] was the first major work on thermal inactivation of spore formers and this was followed in 1922 by the definitive work of Esty and Meyer on thermal destruction of *Clostridium botulinum*.[49] These early quantitative studies enabled the lethal effects of different time/temperature combinations to be calculated and laid the foundations for safe processing of canned foods. It was soon discovered that the type of medium and incubation conditions used for recovery after heating had a pronounced effect on the apparent number of spores that survived the process. This had obvious implications for thermal processing calculations and stimulated much work on optimizing recovery conditions and on understanding the reasons why some media performed better than others.[40,195] General aspects of spore injury and recovery were discussed by Gould[58] and Foegeding and Busta.[52] Other reviews deal with heat injury,[1,11] chemically induced injury,[23] and spore DNA damage and repair.[164] Practical aspects of the recovery of spores damaged by heat, ionizing radiation, and ethylene oxide were reviewed by Roberts.[151]

Typical symptoms of spore injury include increased germination lag and microlag, altered nutritional requirements, sensitivity to recovery on different batches of media, altered optimum temperatures for recovery, enhanced recovery in the presence of starch or activated charcoal, recovery more markedly influenced by gas atmosphere or redox potential, and sensitivity to sodium chloride and other food preservatives and antibiotics. Injured spores thus resemble injured vegetative cells in being more fastidious in their growth requirements than uninjured forms. However, some forms of injury are associated with specific spore structures and are thus unique to spores. Also, the dehydrated state of the core can affect the conformation of macromolecules and hence the nature of damage caused by deleterious agents. This is especially true of DNA and the damage caused by UV light (see below). A further difference is that the spore is metabolically inactive, and sublethal injury is only detectable during germination or outgrowth. Table 15-5 summarizes the symptoms and possible mechanisms of spore injury.

15.5.2 Germination Systems

In some species of *Clostridium* and *Bacillus*, the germination system is apparently more sensitive to

Table 15-5 Sublethal Injury of Bacterial Spores

Treatment	*Manifestation of Injury*	*Proposed Mechanism/Site of Injury*
Heat	Requirement for non-nutrient germinants (lysozyme, Ca-DPA)	Inactivation of germination system
Heat	Altered optimum temperature for recovery	(a) Selective activation/inactivation of germination systems having different temperature optima (b) Need for low metabolic rate to allow coordination of biosynthesis while repairing damage during outgrowth
Heat	Altered nutritional requirements	(a) Stimulation of secondary germination system (b) DNA damage and increased frequency of auxotrophic mutants
Heat, ionizing radiation	Increased sensitivity to salt, nitrite, and some surface active compounds and antibiotics	(a) Damage to spore protoplast membrane (b) Sensitivity of DNA repair enzymes to salt
Ionizing radiation	Loss of heat resistance	Damage to spore cortex
Treatment with alkali and/or reducing agents	Increased sensitivity to iodine, halogen-releasing compounds, chloroform, and, depending on species, hydrogen peroxide, ethanol, and glutaraldehyde	Removal of, or damage to, spore coat
Heat	Increase in germination lag and microlag	Decrease in specific activity of cortex lytic enzyme
Acid	Dormancy Loss of heat resistance	Removal of cations from key sites

heat than the spore as a whole. Spores that have been heat damaged fail to respond to normal germinants and remain dormant unless the germination system is bypassed by exogenous germinants such as lysozyme[33] or calcium dipicolinate.[47]

The beneficial effect of lysozyme on recovery of heat-stressed spores can be very large for some species.[102] For example, the number of survivors recovered after heating spores of nonproteolytic strains of *C. botulinum* at 85°C for 10 minutes was increased by factors of 10^3 to 10^6 if media contained as little as 625 units lysozyme ml^{-1}.[134] Survivor curves obtained using lysozyme-containing recovery media were biphasic, the initial decline representing inactivation of the fraction of spores that were impermeable to lysozyme (Figure 15-6). If spores were treated with alkaline thioglycollate to disrupt the spore coat and render all spores permeable to lysozyme, the initial rapid decline disappeared and linear curves were obtained with the same slope as the "tail" region of biphasic curves. The difference in slope of the two parts of the biphasic curve presumably represents the different rates of inactivation of the germination system and other targets of heat inactivation. On this basis, the germination system of *C. botulinum* 17B is at least 100 times more heat sensitive than other (unknown) critical targets of heat inactivation. Recovery of proteolytic strains of *C. botulinum* is only marginally improved, if at all, by lysozyme, so their germination systems are apparently more effectively protected against heat. The persistence of an active L-alanine germination system in heat-killed spores of *Bacillus terminalis*, was described by Roberts.[151] A shift in the optimum temperature for recovery of *B. subtilis* A from 45°C to 32°C was attributed to inactivation of the normal L-alanine germination system accompanied by activation of a previously latent system active at lower temperatures.[1]

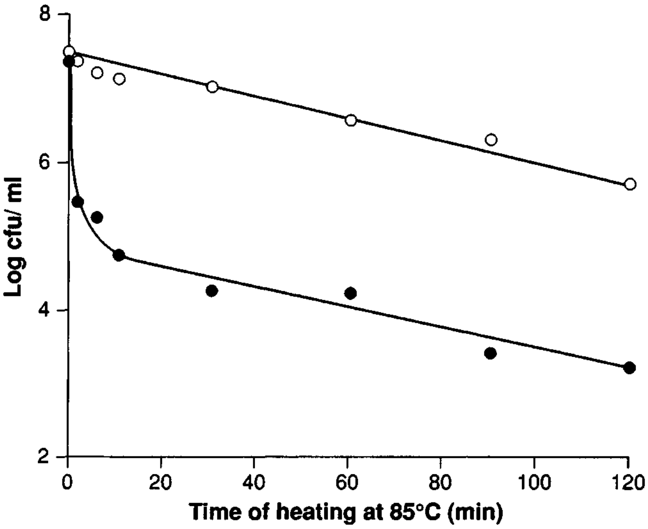

Figure 15-6 The Effect of Treatment of Heated Spores of Nonproteolytic *Clostridium botulinum* Strain 17B with Alkaline Thioglycollate, prior to Plating on Medium containing Lysozyme. (○) cells treated with thioglycollate; (●) cells not treated with thioglycollate. *Source:* Reprinted with permission from M.W. Peck, D.A. Fairbairn, and B.M. Lund, Factors Affecting Growth from Heat-Treated Spores of Non-Proteolytic *Clostridium Botulinum*, *Letters of Applied Microbiology*, Vol. 15, pp. 152–155, © 1992, Blackwell Science Ltd.

15.5.3 Spore Membranes

The increased sensitivity to curing salts, detergents, and antibiotics in spores damaged by heat or ionizing radiation is attributed to damage to the cortical and/or protoplast membranes.[1] Ions or charged molecules do not readily pass across lipid membranes and the greater sensitivity of spores to NaCl, sodium nitrite, and H^+ ions would be consistent with membrane damage sustained in the spore but manifest during germination and outgrowth. Increased sensitivity to antibiotics and detergents has also been attributed to membrane damage because sensitivity was associated with osmotic fragility, and could be repaired in the absence of new macromolecular synthesis. The inhibitory effects of antibiotics and salts were not additive, suggesting that sensitivity to each resulted from damage to a common target site believed to be the membrane. Spores treated with glutaraldehyde, iodine solutions, or chlorine-releasing agents became sensitized to anionic or cationic surfactants and polymyxin. These agents are known to cause damage to spore integuments but the precise site of injury responsible for sensitivity is not yet known. Repair and recovery of resistance occurred after germination at the outgrowth stage.[193,194] Inconsistent or poor recovery of damaged spores on rich nutrient media is usually attributed to the presence of unspecified inhibitors, which can sometimes be removed by adsorption to the amylose component of starch or by activated charcoal. Traces of unsaturated fatty acids, which inhibit germination, are believed to be responsible for inhibition in some cases but the identity of the inhibitors is generally not known. Whether or not spore membrane damage accounts for increased sensitivity is also not clear.

15.5.4 Spore Coat

Damage to the proteinaceous spore coat results in sensitivity to peroxide, solvents, and biocides, but conflicting results and differences between strains have been reported.[23] This form of damage is associated with chemical treatments with alkali, reducing agents, or denaturants rather than the physical treatment used in food processing.

15.5.5 Cortex

Glutaraldehyde can induce a form of sublethal injury in which spores cannot germinate unless first treated with alkali.[41] It is believed that the bifunctional agent glutaraldehyde cross-links amino groups of the cortex, producing a tough sealed structure that prevents normal germination.

Sublethal damage to the cortex is also produced by ionizing radiation. In this case, the observed effect is a decrease in heat resistance resulting from a loss of ability to maintain the dehydrated state of the core. Heating irradiated spores at reduced water activity restored heat resistance.[56] High concentrations of nitrite under acidic conditions can induce germination-like changes in spores. This is caused by hydrolysis of the cortical peptidoglycan by nitrous acid.[58]

15.5.6 DNA

Bacterial spores have been reported to survive for hundreds or, in some cases, thousands of years. The DNA must therefore be in a state that is resistant to the degradative processes—principally hydrolytic and oxidative—that would otherwise destroy it over these time periods, even at moderate environmental temperatures.[99] A large body of evidence has shown that the small acid-soluble proteins (SASPs) that saturate the spore protoplast play a major role in protecting DNA against heat, oxidative attack, freeze drying, and UV radiation.[165] These proteins bind to DNA and protect it against the depurination and strand-breakage events associated with heat or oxidative attack. When SASPs bind to DNA they alter its conformation to a form having some similarities to the A form seen in dehydrated DNA fibers. One consequence is that the photoproduct arising when spores are irradiated with UV light differs from that in vegetative cells (thyminyl thymine adduct, or spore photoproduct, rather than cyclobutane dimer). The spore photoproduct (SP) is potentially lethal, but the lesion is corrected during germination and outgrowth by two repair pathways, nucleotide excision repair, and an SP-specific enzyme called SP lyase. These pathways, however, were not efficient at repairing damage caused by solar radiation.[199]

When spores are heated at very high temperatures (130°–155°C), particularly in the dry state, the protective mechanisms are no longer adequate to prevent depurination damage to DNA. The surviving spore population contains a high proportion of auxotrophic and other mutants, accounting for the altered growth requirements of spores surviving ultrahigh temperature processes.[200] High mutation frequencies are not observed in survivors heated in water at lower temperatures or those exposed to hydrogen peroxide. However, mutants lacking SASPs do show high mutation levels given similar treatment, indicating that the SASPs normally protect DNA against this form of sublethal damage.

15.6 RECOVERY OF INJURED SPORES

Unfortunately, it is not possible to make general recommendations suitable for all types of spore injury or all species, but inspection of the literature will usually yield media and incubation conditions appropriate for the major foodborne spore formers.[82] For recovery of severely damaged spores, additives such as blood, charcoal, pea infusion, or starch to neutralize traces of inhibitors present in the media may be necessary for maximum recovery. In general, media made from fresh infusions have proved better than those made from dehydrated ingredients. Injured anaerobes seem particularly sensitive to gas atmosphere and/or redox potential but the interactions between gas atmosphere and reducing agent can be complex, and in some cases, the simple expedient of pour plating and incubating anaerobically can give better recoveries rather than attempting to find optimum atmospheric conditions for surface-plated samples.[151] Bicarbonate is not germinative alone but at concentrations of 30 mM strongly stimulates germination of several species of clostridia, and may be an absolute requirement for some. Spores of bacilli are not normally affected. Addition of 0.1% bicarbonate to Reinforced Clostridial Agar increased the recovery of heat-damaged spores of *C. botulinum*, *C. sporogenes*, and *C. histolyticum* by between 4- and 130-fold.[151] The optimum temperature and pH for recovery of damaged spores may differ appreciably from the respective optima for growth but there are marked differences in results obtained by different workers depending on the species or strain of organism and the nature and severity of the damaging treatment.

Because germination microlag times vary within populations of injured spores, the apparent probabil-

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ity of survival will depend on the incubation time of recovery media. Similarly, the probability of growth in a product will depend not only on the absolute stochastic probability of spore survival but also on storage time and a factor associated with the statistical distribution of microlag times within surviving spores. Spoilage of food products by thermophilic anaerobes can sometimes occur after a delay of several months. Spore germination times can approximate to a log-normal or skewed log-normal distribution with the mean germination time and standard deviation depending on temperature.[20,59,152] The variation in microlag times within populations of *C. botulinum* would explain why time to toxin production becomes much more variable as inoculum size decreases. In predictive modeling studies of survival and growth of *C. botulinum,* it is normal to use extended incubation times of 3 to 6 months to allow for delayed spore germination. Information on the effect of type of treatment and its severity on the distribution of spore germination times would help refine such models.

15.7 RECOVERY OF INJURED VEGETATIVE CELLS

15.7.1 Choice of Recovery Medium

The most appropriate recovery medium can only be found by trial and error. In general, cells injured by physical or chemical treatments recover best on a rich medium containing blood, catalase, or pyruvate to circumvent problems of oxidative stress. Recent work has shown that strictly anaerobic conditions may be necessary for maximum recovery of heat-treated cells. Starved cells, especially those from marine or freshwater samples, may recover better on a medium containing low levels of nutrients.[53] In some instances, liquid media may give better recovery than solid, possibly because of inhibitors present in agar or desiccation effects. Some species such as *C. jejuni/coli* seem especially sensitive to the dryness of agar plates and a moist atmosphere is important in recovery. (For further information on the choice of recovery medium, see Chapter 62.)

15.7.2 Sample Handling

Sample preparation and dilution can injure delicate cells or kill those already injured. When cells are in the exponential phase of growth they are particularly susceptible to all forms of mild stress.[141] The effects of rapidly cooling growing cells to 0°–5°C can be lethal, but it is not generally appreciated that much smaller temperature shifts can injure exponentially growing cells.[104,107] Many of the commonly used buffers and physiological salts solutions are rather harmful to injured cells and many studies have concluded that diluents should include magnesium (5 mM) and/or peptone (0.1%) to protect susceptible cells.[121] Rapid rehydration of dried samples can be lethal. A slow reconstitution procedure doubled the number of *Salmonella*-positive samples when examining dried milk, egg yolk powder, and other dried foods.[185] The recovery of airborne bacteria was improved by including betaine in the sample diluent.[114]

15.7.3 Resuscitation

There are three general strategies for avoiding counting errors arising from the use of selective media: (1) avoid the use of selective media altogether, (2) formulate the medium to minimize any inhibitory effects on injured target organisms, and (3) perform a resuscitation treatment to allow repair of injury before inoculation on/into a selective medium.

An example of the first approach is the use of size selection with membrane filters to isolate campylobacters and other small microbes.[65] This technique depends on the ability of small vibrioid cells to pass through the 0.45 µm pores of a membrane filter placed on nonselective agar. When applied to stool samples of animals and humans this method allowed isolation of *C. upsaliensis* and other species that were initially overlooked because they were inhibited by the usual selective media.[90,101]

Another possibility is to use selective media that are not inhibitory to injured target organisms. Baird-Parker agar supports good recovery of *S. aureus* cells damaged by drying, freezing, or heating.[9] There are few other examples of such media, presumably because resistance to many selective agents (eg, bile salts, sodium chloride, triphenylmethane dyes, large hydrophobic antibiotics) depends on having intact cell membranes, and these are almost always a site of cellular injury. Mannitol egg yolk polymyxin agar for *B. cereus* and Skirrow's antibiotic blood agar for *Campylobacter* have also been reported to have minimal inhibitory effects though selectivity may be less as a consequence.[121]

Where inhibitory selective media cannot be avoided, resuscitation on solid or in liquid is needed. In addition to improving the sensitivity of detection methods, including a resuscitation step tends to improve consistency and to reduce variability between laboratories.[185] Solid medium repair is the method of choice where quantitative estimates of viable numbers are needed. This can be achieved by spreading cells on the surface of a nonselective agar medium and, after allowing for repair to occur, overlaying with a selective medium. A more satisfactory method is to incubate cells on a membrane filter on a nonselective medium before transferring the membrane filter to selective agar, as in the modified direct plate method for *Escherichia coli.*[73] The increase in count achieved by solid medium repair typically varies between 2- and 100-fold when enumerating stressed coliforms in food.[121,147]

In presence/absence tests designed to detect very low numbers of cells, as used with *Salmonella*, a pre-enrichment treatment in liquid medium for *ca.* 18 to 24 hours allows low numbers of cells to repair injury and also to multiply to levels that can be detected during the later stages of the procedures. Recent work has revealed wide differences in the ability of pre-enrichment broths to recover injured salmonellae. The number of cells recovered differed by as much as 1,000-fold between the worst and best media[173] (see also Chapter 62).

15.7.4 The Time Needed To Repair Injury

The time required for repair depends on the organism, the type and severity of injury, and the inhibitory nature of the selective agent. Figure 15-7 shows that lag times of *Salmonella typhimurium* injured by heat or gamma-radiation increased as the surviving fraction decreased, but the lag times of heat-injured cells after equivalent lethal treatments were much longer. The implication of these results is that lethal and sublethal injury occurred at different relative rates depending on the treatment. In the case of heat, there was presumably much more "collateral" damage to nonlethal targets before cells died. With gamma-radiation, which has a much more specific target, such damage was substantially less. A model of thermal injury was presented by Gould[60] in which heat is assumed to inactivate a key lethal target, while at the same time destroying many other targets present at higher copy numbers. Loss of the less-critical targets does not cause death until their numbers are reduced to very low numbers or the cell is subject to additional stress. A modified version of this model is shown in Figure 15-8. Critical and less-critical targets are present at different copy numbers per cell as before, but the probability of sustaining a "hit" is independent for each target and depends on the nature of the damaging treatment (heat, acid, radiation, etc). Lethal and sublethal events thus occur at random, but with a different probability for each. If the probability of a lethal event is much lower than that of a sublethal event, surviving cells will be severely damaged and will have long lag times. If the probabilities

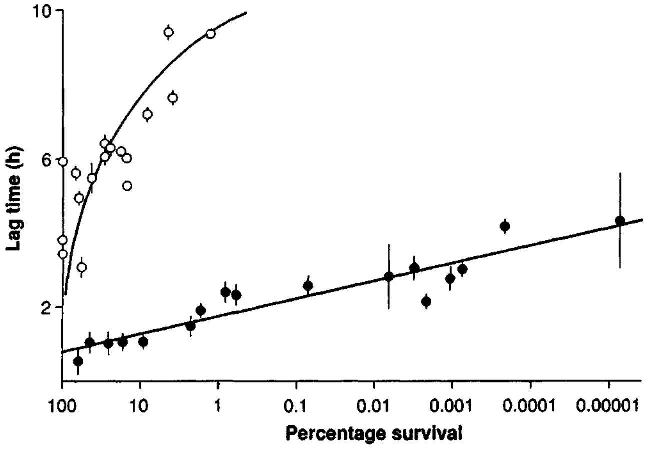

Figure 15-7 The Effect of Heat or Gamma-Radiation on the Duration of the Lag Phase of *Salmonella typhimurium*. Lag times were determined at different levels of survival following heating in 0.1 mol l^{-1} phosphate buffer pH 6.0 (○) or following gamma-irradiation from a ^{60}Co source (●). *Source:* Reprinted with permission from B.M. Mackey and C.M. Derrick, A Comparison of Solid and Liquid Media for Measuring the Sensitivity of Heat-Injured *Salmonella typhimurium* to Selenite and Tetrathionate Media and the Time Needed to Recover Resistance, *Journal of Applied Bacteriology*, Vol. 53, pp. 233–242, © 1982, Blackwell Science Ltd.

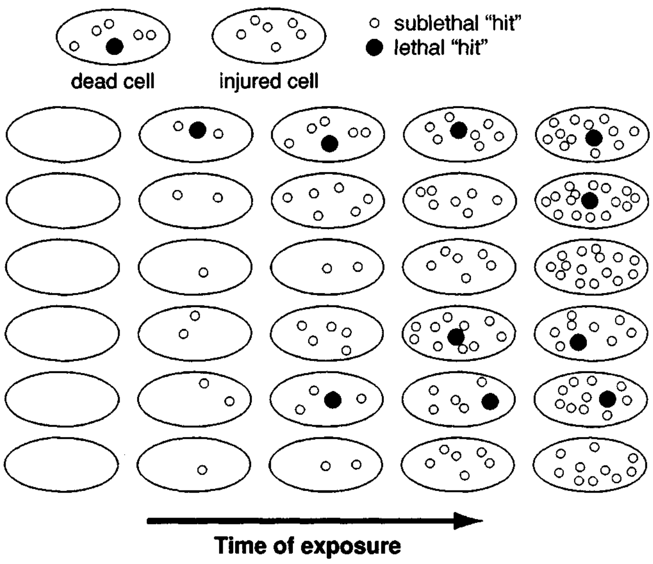

Figure 15-8 Random Accumulation of Lethal and Sublethal Injury in a Bacterial Population. *Source:* Copyright © 1989, G.W. Gould.

are similar, survivors will be less severely injured and lag times correspondingly shorter.

Measurements of repair times of cell populations using the differential plating method suggest that, under optimum conditions, injury caused by chilling, freezing, freeze drying, gamma-radiation, or acid is normally repaired within 4 to 5 hours and often within 1 to 2 hours. Mild heat injury can be repaired in about 5 hours under optimum conditions but cells with severe heat injury may require much longer times. *Enterococcus faecalis* needed up to 24 hours to recover resistance to tellurite after being heated at 60°C for 4 minutes.[133]

15.7.5 Heterogeneity within Injured Populations

The differential plating method gives an estimate of the "average" repair time of a population of cells and is therefore biased toward the fraction of cells in the population with the shortest individual lag times. These quick-repairing cells will multiply and mask the presence of cells having longer repair times. A mathematical treatment of the relationship between the lag time of a population, and the distribution of lag times of single cells within the population, was provided by Baranyi.[10] Pathogens in food may be present in low numbers and unevenly distributed. When estimating the risk of an injured cell recovering within a particular time, it is therefore important to understand the distribution of lag times within a population. The repair of single cells of *S. typhimurium* was monitored by spreading heat-injured cells on membrane filters, incubating these on nonselective agar to allow repair of injury and transferring at intervals to nutrient agar plates containing salt. This revealed that in a population having an "average" lag time of 9 hours, some cells required up to 14 hours to repair injury.[104] The distribution of lag or repair times may also be examined by progressively diluting a culture and measuring lag times in replicate inocula using an automated apparatus.[104] If the culture is diluted so that only 63% of replicate inocula will result in growth, then the average number of cells per inoculum is one. Use of this approach revealed a very wide distribution of lag times of single cells of heat-injured *S. typhimurium*, with many values in excess of 20 hours[173] (Figure 15-9). These observations have obvious implications for pre-enrichment methods intended to allow resuscitation and detection of low numbers of injured cells (see also Chapter 62).

15.7.6 The Relevance of Resuscitation to Newer Methods of Detection

A large number of alternative methods for detecting microbes are now available with test times as short as 30 minutes. Broadly, these methods are based either on detecting the physical presence of a cell (eg, gene probe or antibody-based methods) or on detecting metabolic activity (eg, electrochemical or dye-reduction methods). The role of resuscitation and/or pre-enrichment in this context is (1) to enable target organisms to increase to detectable levels or (2) to allow restoration of metabolic activity lost as a result of stress. Resuscitation would obviously play no role in the detection of preformed toxins or nucleic acids directly extracted from food.

Rapid methods are often inherently less sensitive than traditional culture methods where self-amplification (multiplication) of the target occurs. Even where a method is very sensitive, as with the polymerase chain reaction (PCR), its sensitivity in practice is limited by the problems of extracting the target analyte from the food matrix and presenting it to the endpoint detection system. With liquid samples concentration of target can be achieved by filtration but in more complex food matrices it is not possible to remove and concentrate very low numbers of cells. Direct extraction of DNA from cheese, coleslaw, and raw chicken followed by PCR allowed detection of 10^3–10^4 cfu of *Yersinia enterocolitica, Aerococcus viridans*, and *Listeria monocytogenes* per g.[45] To increase sensitivity, therefore, requires pre-enrichment, as with conventional methods. However, because gene probe or antibody-based methods are highly specific, it should be possible in principle to examine pre-enrichment broths directly, without the need for selective enrichment. Many protocols employing nonselective enrichment followed by PCR or fluorescent antibodies have now been reported[96,100,137,196] but there appear to be few studies on the effect of cell injury on the population dynamics in these systems and on the resuscitation times and conditions needed to achieve detectable levels.

Gene probe or antibody-based methods do not distinguish between live and dead bacteria, and there has been considerable interest in the use of fluorogenic indicators of metabolic activity or membrane permeability of single cells.[42,44,128,184] When used in combination with labeled antibodies, these stains can give an indication of the viability of specific organisms in a mixed flora.

However, metabolic activity and membrane permeability can be transiently affected by cell injury, and this will affect the response of cells to the different probes. The apparent absence of metabolic activity could mean either that a cell is dead or that it is injured or otherwise metabolically quiescent, and resuscitation is required to distinguish between the two states. The ability to reduce tetrazolium salts was temporarily lost when cells of *Listeria monocytogenes*

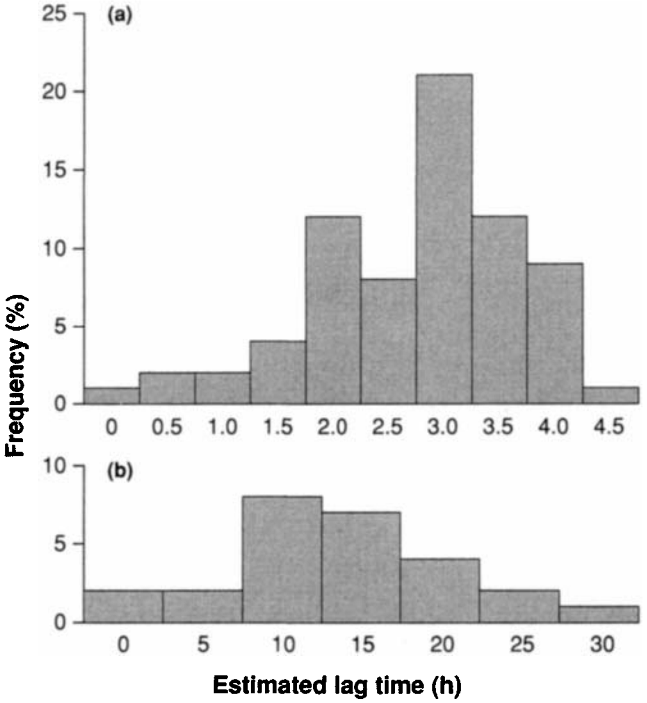

Figure 15-9 Distribution of Lag Times in *Salmonella typhimurium.* **A**, unheated cells; **B**, heat-injured cells. *Source:* Data from P.J. Stevens et al., The Use of an Automated Growth Analyzer to Measure Recovery Times of Single Heat-Injured Salmonella Cells, *Journal of Applied Bacteriology,* Vol. 83, pp. 445–455, © 1997, Blackwell Science Ltd.

were exposed to mild heat stress, but this capacity was restored during resuscitation in liquid medium.[27]

A recently described method for detection of *S. typhimurium, Pseudomonas aeruginosa,* or *C. jejuni* employs replication of a species-specific bacteriophage as an amplification strategy.[118] Selective media are unnecessary in this method but whether sublethal injury would affect the ability to adsorb or propagate phage is not yet clear. Messenger RNA has a half-life of only a few minutes in bacterial cells and its presence has been suggested as an indicator of viability.[14,91,166] It is likely that the presence of some mRNA types would temporarily disappear as a result of injury, in which case resuscitation would be necessary before probing.

15.8 THE ROLE OF SUBLETHAL INJURY IN COMBINED TREATMENTS FOR PRESERVING FOOD

The maintenance of homeostasis requires an intact semipermeable cell membrane and the expenditure of energy. Therefore, any treatments that disrupt membranes or interfere with energy conservation will hinder or abolish the homeostatic capacity and will eventually lead to degeneration and death. If membrane structure or function is disrupted, even relatively mild environmental stress can prevent growth or kill the cell. Fruitful strategies for combined treatments based on sublethal injury might therefore aim to (1) damage membranes, (2) impose an external stress, and (3) arrange conditions such that repair of injury is delayed or prevented.

Most physical stresses such as heating, freezing, high-pressure treatment, dehydration, and high-intensity electric fields cause damage to both outer and cytoplasmic membranes of Gram-negative bacteria and also to the Gram-positive wall and membrane. Examples of combined treatments that probably involve membrane damage are heating at low pH; high pressure and heat; and heating with ultrasound energy and a slightly raised hydrostatic pressure.[46,61,62] Physical stresses, such as exposure to high pressure or treatment with chelating agents, damage the Gram-negative outer membrane and sensitize cells to nisin and lysozyme, thus offering possibilities for use in combination treatments.[67,84] If repair processes are slowed, for example, by holding at low temperature, or additional metabolic burdens imposed, cell death ensues albeit slowly.

In calculating safe food processing treatments, it is normal to specify a certain number of log reductions that must be achieved, eg, 6 to 7 for organisms like *Salmonella.* The use of combination treatments effectively increases the death rate either by enhancing lethality during the treatment itself, or by ensuring that any injured survivors subsequently die on continued exposure to inimical external conditions (Figure 15-10). Because there is a spectrum of degrees of injury in surviving populations, the probability of growth under restrictive conditions will depend on the particular distribution of injury within surviving populations. There appears to be no theoretical treatment of this.

The limiting factor in applying mild combination treatments is often the presence of bacterial spores, particularly of psychrotrophic clostridia. However, some combined treatments such as ultraviolet light combined with hydrogen peroxide, heat, and acid, and heating followed by ionizing radiation are effective even against spores.[187] There is some evidence that damaged spores may die if held at ambient temperatures under some conditions that do not permit growth; this requires further investigation.[98] The application of combination treatments in food preser-

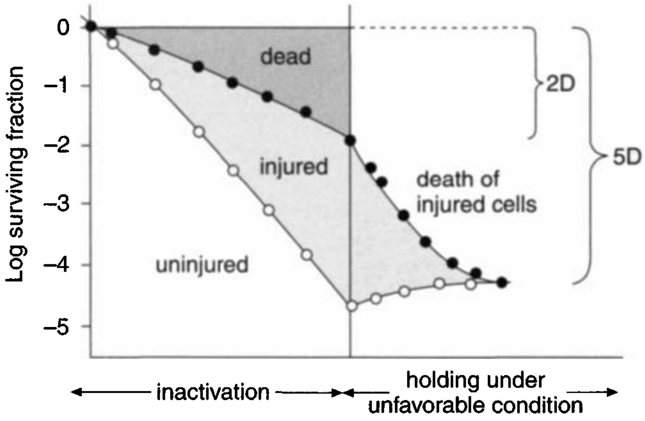

Figure 15-10 Effect of Preventing Repair of Sublethal Injury on Inactivation of Microbes. If repair of injury is prevented by unfavourable post-treatment conditions, the effective extent of inactivation is increased from 2 to 5 log units reduction in this example.

vation is not new, and many traditional foods such as salami-type fermented sausages and pasteurized cured meats rely on combination treatments for their safety and stability. However, many new combinations are being tested and our understanding of the physiological mechanisms involved is still rudimentary. A better knowledge of the role of sublethal injury in combined treatments would help in the development of new preservation methods that provide improved quality while maintaining product safety.

15.9 CONCLUDING REMARKS

The phenomenon of sublethal injury in bacteria has been recognized for over 50 years, and it is now possible to explain, in general terms at least, many of the altered phenotypic properties of such cells in terms of injury to particular cell structures, as discussed in this review, although detailed physiological explanations are often lacking. The VBNC state has been recognized more recently and continues to excite controversy. The following questions often arise. "Do VBNC cells really exist?" "Are they more resistant than normal cells?" "What should the food microbiologist be doing about them?"

If one accepts that bacterial viability implies the capacity to multiply under suitable conditions, then the mere detection of metabolic activity in intact cells (eg, the capacity to reduce INT or elongate in response to nutrients) is not sufficient grounds for assuming they are alive. In a few cases (eg, starved *M. luteus*) the viability of cells that are temporarily nonculturable (or fastidious to use the terminology suggested here) has been demonstrated by applying a resuscitation treatment that restores full culturability. However, for most organisms described as VBNC, unequivocal resuscitation data are lacking and quantitative estimates of numbers of VBNC in populations of cells are almost nonexistent. This does not mean that VBNC cells of the common pathogens do not exist in food, it simply means that the case has not been rigorously proven and that it is not yet possible to gauge the extent or significance of the phenomenon. Given the long periods over which membrane integrity and metabolic activity can persist in nonculturable cells, it would be particularly helpful to have comparative information on the longevity of such functions in unequivocally dead cells, eg, those killed by radiation.

The distinction between injured and "nonculturable" states was originally based on perceived differences in the causes of these conditions and in the associated physiological characteristics. Sublethal injury was usually associated with exposure to physical or chemical stress and was characterized by the loss of ability to grow on selective, but not on nonselective media. VBNC cells are more typically associated with starvation states, and fail to grow even on nonselective media. These distinctions have become blurred because the term VBNC is now also applied to cells produced by sudden physical insult, such as aerosolization, that need betain for recovery (whereas salmonellae in milk powder that need slow rehydration to recover are called injured). Differences based on physiological properties are also less clear with the recognition that injured cells also may fail to grow on rich nonselective media, eg, because they are sensitive to oxidative stress.

The risk posed by different pathogens in foods, and therefore the stringency of sampling schemes and detection methods, depends on numerous factors including the infective dose, the severity of illness caused, whether subsequent food handling will increase or decrease the risk, the likelihood of spread within the community, and exposure of susceptible groups. Clearly the presence of highly infective pathogens, even injured ones, in foods that are to be consumed without further treatment is considered unacceptable, particularly where susceptible groups in the population are at risk or if there is a chance of further microbial growth in the food. In such cases, it is very important to detect even severely injured cells. Current resuscitation methods may not allow full recovery of injured cells that require strictly anaerobic conditions for repair or those needing very long repair times. To rectify these possible deficiencies would present substantial practical difficulties for routine laboratories. Therefore, the importance of anaerobiosis in recovering injured bacteria and the significance of severely injured cells need clarifying.

A better understanding of the distribution of properties in populations of stressed cells would allow the effect of cell injury to be included in risk assessment exercises, and would provide a basis for estimating the lethality of combined processes that depend on inhibiting sublethally injured cells. Experimental and theoretical approaches to studying the distribution of lag and repair time within bacterial populations have already begun,[10,173] but corresponding stochastic approaches to other relevant properties of injured cells, such as resistance to acid, heat, and food preservatives, or virulence, have not been attempted. With the advent of flow cytometry and cell sorting, the methods for approaching the problem of individual cell variation are now available.

Understanding the basis of the fastidious phenotype (ie, extreme sensitivity to recovery conditions) is a central question for microbial physiologists and geneticists interested in microbial survival. Only when we know the underlying mechanisms will we be able to decide whether VBNC cells represent a class that is distinct from injured cells and whether they are more resistant or require a different approach to recover them.

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16 Principles and Application of Predictive Modeling of the Effects of Preservative Factors on Microorganisms

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16.1 INTRODUCTION

In the first part of this chapter, the history and progress of an area of food microbiology known as *predictive microbiology* are summarized along with guidelines for the efficient acquisition of data for effective modeling. In the second part, mathematical aspects of modeling are addressed. Finally, topics that require further research effort if modeling is to be used more widely as a support tool in food microbiology are identified.

The safety and shelf life of food products have traditionally been estimated by challenge tests, or inoculated pack experiments, the food being inoculated with the microbe(s) of concern, and stored under representative, and often abuse, conditions. After storage, the food is tested microbiologically to determine whether the inoculated microbes have multiplied, survived, or lost viability. Retrospectively, some account is taken of properties of the food such as pH and percent salt-on-water or water activity (a_w), and the temperature of storage. Much reliance has been placed on such relatively crude tests, although the circumstances of the challenge test often differ substantially from the range of conditions that may be experienced during food processing and storage. Care must be taken to ensure that introducing the microbial challenge into the food does not change the local environment, eg, by introducing water with the inoculum, thereby facilitating local microbial growth that would not have occurred if water had not been added. It is common practice to use a relatively large inoculum in challenge tests using pathogenic bacteria, commonly 10^3–10^6, justified as giving the results "a margin of safety." However, assurance that such a large inoculum mimics the behavior of a smaller number of pathogens is weak. Such high numbers are not representative of the numbers of pathogens normally present in raw and processed foods, and there is evidence that growth can occur more readily from high numbers than from low numbers.[65] "Predictive microbiology" aims to overcome many of the disadvantages of challenge testing, particularly to allow the effects of combinations of factors on microbial growth and survival to be mathematically modeled.

16.2 OVERVIEW

The history of predictive microbiology was reviewed in admirable detail by Ross and McMeekin[71] who traced its origins to a number of initiatives. These initiatives included the following: research on fish spoilage in the United Kingdom (UK)[76] and Australia[55]; recognition that even so-called "rapid" microbiological methods were still relatively slow to provide the essential information whether microbes grew or died[64]; attempts in the United States (US) to define combinations of factors that would prevent pathogen growth and toxin formation[31]; and work in Canada to draw together the results of many apparently disparate experiments on *Clostridium botulinum*.[36] The ready availability of powerful personal computers and user-friendly software that encouraged microbiologists to explore areas of statistics and mathematics also contributed. Those uncoordinated interests, together with attempts to define the boundaries of "growth–no growth" of pathogens[62,63] led to results of complex experiments being expressed as equations that predict the probability of growth and toxin production in *C. botulinum*.[14,26,68]

The development of predictive microbiology received a huge boost after the UK Ministry of Agriculture Fisheries and Food reviewed possible topics for new research programs and decided, in 1988, to fund a coordinated program on bacterial pathogens to:

1. obtain systematic data on microbial growth and death for different temperatures, pH values, water activities, and preservatives
2. treat the data mathematically to make models of the microbes' behavior
3. ensure that the models mimic the microbes' behavior in a range of growth and death conditions
4. "validate" the model by comparison with independently published scientific data or by selected challenge tests.

At the time such targets were ambitious. There was no doubt that data could be generated, but experience of modeling such data was limited. There was much argument about whether models produced from laboratory experiments in media would be relevant to microbial growth in foods, or whether models should be produced from growth data in the foods in question. The background microbial flora was also believed to play some role in determining the rate and extent of growth of pathogenic bacteria.

In 1992, the outputs of that research program led to a "bureau service" based on the database and models derived from it. However, it was a somewhat cumbersome procedure and did not prove popular with the food industry. The bureau service was replaced in 1994 by the commercially available software Food MicroModel, running under Windows™. Work was also initiated in the US where the US Department of Agriculture undertook a similar program, modeling the growth and death responses of bacterial pathogens, leading to the software Pathogen Modelling Programme,[15] initially available running under DOS, and the most recent version under Windows™.

Interest in predictive microbiology in other European countries was fostered under a Food Linked Agricultural and Industrial Research (FLAIR) Concerted Action program on "Predictive Modeling of Microbial Growth and Survival in Foods" (CA No. 5, COST 905), and a FLAIR Shared Cost program on "Computer-Aided Process Design Procedures to Improve Quality and Safety of Products with a Limited Shelf-Life" (AGRF-DT91-0047), each from 1989 to 1993. The network of scientists in those programs has been maintained and expanded through COST 914 "Predictive Modeling of Microbial Growth in Foods" (1994–99), within which four subgroups have been established to explore topics identified as important to the improvement and further progress of predictive microbiology viz.:

1. validation of predictive microbiology in a wide range of European foods
2. instrumental methods for data capture for advanced predictive microbiology
3. exploration of mixed population effects in predictive microbiology
4. modeling microbial survival to eliminate pathogens from foods.

16.3 MODELING MICROBIAL RESPONSES

16.3.1 General Considerations

Challenge tests have long been recognized as expensive, slow, and demanding on facilities and microbiological skills, and as providing only modest assurance that the microbial behavior in the challenge test would be repeated in the food product during distribution and sale. Knowledge acquired in challenge tests is not cumulative, and when a product formulation, packaging, or distribution system changes, challenge tests must be repeated. The literature is witness to each new bacterial hazard being investigated in a variety of foods and a range of environmental conditions. Despite the vast amount of information on the growth and death responses of bacterial pathogens, no serious attempt had been made to bring that information together in a concise and readily usable form, until that by the International Commission on Microbiological Specifications for Foods (ICMSF).[38]

An alternative to challenge tests would be to understand sufficiently the responses of the microbes of concern to the main controlling factors in the food environment, to build a cumulative store of knowledge, and to develop the means of interpolating calculated microbial responses. Microbiologists have long dreamed of being able to model the growth and death of microbes in foods. The prospect of not having to repeat challenge tests when the responses are confidently assured was embodied in the term "predictive microbiology." Before any data were generated within the major programs of research mentioned above, numerous points of debate arose over the experimental approaches, choice of media, choice of organism, etc. Protocols had not been scrutinized widely and agreed, so any advice and comments were based on individuals' particular experience. No assumptions could be made that growth in laboratory media would necessarily mimic confidently growth in foods. Neither was it clear how many factors would need to be considered to enable accurate predictions to be made, so the early experiments tried to identify how much of the microbial response could be explained by the effects of a small number of controlling factors. As work progressed, a number of important principles and guidelines emerged that together led to effective protocols for predictive modeling, as summarized below.

Culture medium: In general, foods are rich in nutrients, so laboratory media chosen for modeling should be rich also, rather than minimal or likely to lead to early nutrient depletion during the growth of cultures. It is unwise to rely on a single medium for all organisms, rather a rich medium should be selected that is designed for the organism and likely to allow growth approaching the maximum possible growth rate.

Choice of organisms: Debate continues whether models should be developed using a single strain of a pathogen or spoilage organism or using several strains mixed in a "cocktail." Using a single strain gives a more precise definition of its responses, but strains of the same species may vary in their response, especially in suboptimal conditions. If a single strain is used, it should have been screened against the conditions to be modeled and have been shown to be representative of other strains of that organism, ie, similarly tolerant of low water activity and low pH, etc, and with similar temperature characteristics. Ideally, recent isolates, for example, from spoilage situations, should be used. Strains held for long periods, for example, in culture collections, may have lost important resistances or other characteristics. Many modelers have relied upon "cocktails" of strains to obtain a response, which takes account of the fact that one strain may grow at a slightly lower temperature, while a different strain may be slightly more tolerant of low pH. By using "cocktails," it is hoped that the model includes all the variations and, most importantly, any extremes of response.

Pathogens: Strains that have been associated with recent outbreaks of illness should be used wherever possible. As with spoilage organisms, strains that have resided in culture collections for long periods should be avoided to avoid losing any important property through repeated subculture. It should be recognized that minor differences in responses may occur within species, eg, in different serotypes of the same organism. However, modeling every important serotype separately is usually an impossible task and the only realistic solution is to include in the "cocktail" representatives of all the important serotypes.

Maintenance of organisms: It is possible that repeated subculture, even during the course of a data generation program, could result in changes similar to those found in old cultures. Data generation usually occurs over a relatively few months and nonessential subculturing should be avoided. However, even when additional data on pathogens have been derived some months later, after the completion of an initial series of experiments, those new data have been found generally to replicate earlier data adequately so that this has not proved to be a serious problem.

Preparation of the inoculum: During the early evolution of growth modeling studies, inocula were prepared in a standard manner, usually growing under near-optimal conditions to reach the exponential phase. Subcultures were then made and placed into a range of growth conditions, some near optimal, others judged to be close to conditions that would not support growth. If the inoculum was prepared by subculturing from conditions identical to the intended growth conditions, a different growth response may result, eg, in experiments to investigate growth at low temperature. In particular, the lag phase is generally most affected. However, the differences between the two responses have not been extensively quantified and most modelers regard the additional complexity of the latter procedure a disincentive. One important consequence, therefore, is that growth rate is usually modeled much better than lag time (see below). While this effect of "preconditioning" prior to subculture is well recognized in growth modeling, it has not been identified in survival modeling, ie, under conditions where growth cannot occur, but where it may also be important.

Changing conditions during growth: A conscious decision is usually taken to model growth, survival, and death as functions of the initial conditions of pH, water activity, preservative concentration, etc., at constant temperature in order to keep experimentation as simple as possible. In more sophisticated modeling, it becomes necessary either to generate models under changing conditions, or to show that models developed under a series of static conditions can be satisfactorily predictive when applied to those that change.

Aeration: When deciding on experimental approaches to the generation of data intended for modeling, aeration of cultures of aerobic bacteria should be considered. Shaking, combined with the use of a small volume relative to the size of the container, appears a simple solution, but rarely achieves good oxygen transfer into a culture as the concentration of organisms, and therefore their oxygen demand, rises. Gassing makes experimentation more complicated and may be justified where the effects of oxygen on growth are known to be substantial. However, it has generally been found that predictions from many existing models that were derived using static cultures appear to mimic adequately the documented responses of bacteria in and on stored foods.

Sampling: Two different approaches have commonly been used: (1) a single culture vessel is sampled repeatedly during storage, or (2) replicate cultures are derived from a single inoculated culture vessel, one being destroyed by sampling at each time interval. Which is used is a matter of personal choice. The former offers a more evenly mixed culture, but suffers from a greater risk of possible contamination, especially if the incubation period is protracted. The latter is effective and flexible as long as a sufficient number of replicates is provided to take account of unexpected slow growth. Either approach must provide a sufficient number of estimates of viable numbers to permit the inflexions in growth curves to be characterized, ideally of the order 12 to 20 estimates per curve.

16.3.2 Modeling Thermal Death

Although some workers regard modeling of microbial responses to food-related environments as novel, food technologists have for many years applied models of microbial death in major food processes worldwide, eg, to ensure sufficient inactivation of spores of *C. botulinum* in low-acid canned foods via the 12-D concept, the minimum heat process to inactivate 10^{12} spores, based on heat resistance studies on spores of *C. botulinum*.[28] This approach, as with all current microbial growth and death modeling, is empirical, interpreting the response of the organism without understanding the underlying mechanisms, in this case, the mechanism of spore death. Similarly, minimum processing times for elimination of *Salmonella* at a range of minimum internal temperatures[35] led to Guidelines and Good Manufacturing Practices[1] addressing the problems of cooking fresh and cured beef with respect to survival of *Salmonella*, and similar approaches have been made with respect to *Listeria monocytogenes*, *Escherichia coli* O157:H7, and others, all based on empirical models of thermal inactivation.

16.3.3 Modeling Growth

Modeling microbial growth has a long history in the fermentation industry, where the objective has been to maximize the generation of biomass by optimizing the effects of growth temperature and the supply of substrate(s). Likewise, in the production of fermented products of milk, meat, fish, and vegetables, rapid growth of the lactic flora and efficient fermentation are essential first steps to providing a wide range of traditional, safe products. In most other circumstances, however, the goal of the food microbiologist is to prevent, or minimize, microbial growth, rather than to optimize it.

Simple growth models have helped the identification of critical limits for microbial growth and aided the development of processing guidelines that give greater assurance of safety, eg, the Food Safety and Inspection Service (FSIS) directive[83] on time/temperature guidelines for cooling heated products is designed primarily to minimize growth of *Clostridium perfringens*. The identified control points, minimum and maximum holding temperatures, and cooling times were based on data derived from a comprehensive study of the growth of *C. perfringens* in chili.[10]

The number of factors that affect the growth or survival of foodborne microbes is large, including temperature, pH and the nature of the acidulant, water activity and the nature of the humectant, oxygen availability, redox potential, preservatives, etc. All of these factors need to be kept in mind when assessing whether a microorganism could pose a problem in a particular food system. The expectation of complex relationships between a large number of controlling factors, demanding huge experiments, had a negative effect on the progress of microbial growth modeling. Investigating all the identified factors at even a few levels would be beyond the resources of most researchers.

Studies of the combined effects of several controlling factors in food systems began more than 25 years ago. In the 1970s, research characterizing the effectiveness of sodium nitrite against *C. botulinum* in model systems and cured meats expanded because of interest in minimizing formation of nitrosamines without loss of antibotulinal activity or shelf life.[2,16,27,63,66] However, little of that research was used to construct models, perhaps due to insufficient systematic data, or lack of availability of, or access to, effective modeling techniques and because computers were not widely available. Nevertheless, that early work clarified the relative importance of the main controlling factors, and identified the need for a modeling approach. An early application of response surface analysis techniques to food microbiology[75] demonstrated that of 16 ingredients in a soy-based ground meat analog, only 4 had a significant impact on the growth of *C. perfringens.*

Probability Models

Hauschild[36] estimated the probability that a single spore of *C. botulinum* would germinate and produce toxin in a food, drawing together results from different authors. Other investigators systematically estimated the effects and interactions of a range of factors on the probability of germination and outgrowth of *C. botulinum.*[3,50] Other probability models have been published for toxin production by proteolytic strains of *C. botulinum* types A and B[36,43,68,87] and growth of *Zygosaccharomyces bailii.*[19]

Although such models indicated only the probability of growth or toxin production under defined conditions of temperature and time, the most significant factors and interactions influencing growth were clearly identified. The probability of growth quickly gives an indication of the safety implications of changes in product formulation or in storage conditions. Conclusions were sometimes unexpected, for example, growth of *C. botulinum* was shown to be unlikely to occur in bacon, which was receiving considerable attention and research, whereas products that had largely been ignored, such as turkey rolls, were less safe.[36]

Kinetic Models

Approaches to estimating the growth kinetics, particularly the lag phase and the maximum growth rate, vary in their mathematical complexity, but have similar aims, eg, the "square root,"[47,59] linear Arrhenius,[23] nonlinear Arrhenius[14] relationships, and the use of cardinal values from the literature.[72] Exploring the effects of several controlling factors at the same time has most commonly modeled derived growth parameters against the controlling factors by a response surface approach.[9,17,32,45]

More recently, dynamic mathematical models have been developed. The advantages and disadvantages of these and some of the potential pitfalls in their use have been discussed.[4,5,49] A simplification of the assumption that the state of a cell population can be defined by the physicochemical environment (temperature, atmosphere, substrate, etc) and the intracellular conditions[29] has been used in a new dynamic model.[4,5] The environment dependence of the maximum specific growth rate and the lag (or their reparameterized forms) have sometimes been modeled independently, although it has long been recognized in practice that there is often a high correlation between them. The new model addresses this problem by taking the product of those two parameters to be constant.[4]

It is important to be able to predict the course of bacterial growth under a time-temperature profile that changes with time. If the temperature remains within the range that allows growth, instantaneous adjustment of the specific growth rate to the temperature changes can be assumed if the cells are in the exponential phase.[11,30,51,52] The new model also makes it possible to predict the course of bacterial growth when the temperature changes in the lag phase during which the cells adjust to a new environment.

A serious hindrance to the furtherance of modeling as an approach has been a deeply rooted belief that the natural "spoilage" flora of a food influences the multiplication of any pathogen(s) present. However, when "predictions" from models developed in laboratory media are compared with measures of growth in foods, and account is taken of the pH, % brine (or a_w), and temperature, the estimates are often very similar. The conclusion that must be drawn is that much of the response of the pathogens can most often be explained by a relatively small number of controlling factors, and that in many instances, the growth response is affected little by the spoilage flora. Similarly, in most instances the composition of the food itself plays a minor role in determining the microbial response, contrary to earlier beliefs that the nature of a food would be a key determining factor.

Predicted growth responses from different models have been compared extensively.[45,78-80,85] The agreements between predictions of growth rate and observed values have, in general, been good. Nevertheless, in some instances the predicted growth responses derived from studies using laboratory media do differ from the observed, published or challenge test, values. The differences are sometimes clearly due to factors not included in the model such as nutritional factors, naturally occurring antimicrobials or added preservatives, for example, nitrite in the food or carbon dioxide in the atmosphere above it. Some foods contain natural antimicrobial systems, eg, eggs.[21] In other cases, the structure of the food plays a role, perhaps by limiting diffusion of substrate or oxygen to the microbe, or acid produced away from it.[12,67] There are examples where the response of a pathogen is influenced by the spoilage flora, in particular by the local change in pH caused by its growth. Several models with more than three factors have been developed to accommodate the additional controlling factors.[17,45]

If the observed response in a particular foodstuff still differs substantially from that predicted by a model containing all the known major controlling factors, it is important to determine the reasons, and to keep a record of them. Some of the problems that arise and need to be addressed are identified in a recent review.[48]

16.3.4 Modeling Nonthermal Inactivation

In foods, there are sometimes conditions of temperature, pH, and a_w under which multiplication of bacteria cannot occur but the conditions are not lethal. Vegetative pathogens may be able to withstand adverse conditions for long periods, often months, without losing their viability. Effort has already been put into modeling those responses.[39,86] Data on survival in adverse conditions are available for *Listeria monocytogenes*,[18,20] *Yersinia enterocolitica*,[42] and *Staphylococcus aureus*[89] but, like the growth models, these models are empirical and, as yet, without any basis in the physiological responses of the microbes during loss of viability or survival.

16.4 COMPUTATIONAL ASPECTS OF CREATING PREDICTIVE MODELS: A NEW MODELING APPROACH

16.4.1 Is a New Modeling Approach Needed?

Although currently used models of microbial growth and survival sometimes fail to predict microbial behavior in particular foods, the reasons for failure, as indicated above, are often known or suspected. Usually, the cause of the failure is not inadequacy of the model, but lack of knowledge of all the important factors in the food. It is therefore not likely that radically new models *per se* will lead to improved predictions, though new approaches, such as that proposed below, may be of some value. For example, although biotechnology has produced many models of microbial growth (see, for example, the excellent reviews of Roels and Kossen[69] or Nielsen and Villadsen[54]), most of those models cannot simply be copied and applied to the different purposes in food microbiology. The reasons for this are as follows: (1) with the exception of fermented foods, the goal of food microbiologists is to minimize or prevent microbial growth rather than optimize it, as is often the case in biotechnology; (2) the cell concentrations of interest in food microbiology, particularly with regard to pathogens, are much lower than in biotechnology, where typically concentrations much greater than 10^8 or so cells ml^{-1} are encountered. As a consequence, some models validated at high cell concentration, eg, turbidity, biomass, or conductance measurements, should not be applied directly without establishing the relationship between the indirect measurement and cell numbers at lower bacterial concentration; (3) in food microbiology, the lag phase and the factors that determine its duration are very important, while it is less so in biotechnology. On the other hand, well-established models of the transition from the exponential to the stationary phase, like Monod's model,[53] lose significance in food microbiology because substrate limitation is rarely important until microbial concentrations reach the very high levels associated with spoilage. Below, the basic elements that underpin most models of microbial growth are summarized. Then a new, and hopefully improved, approach will be developed.

16.4.2 Exponential Growth

The following basic model of population growth has been widely used in biological sciences since Malthus.[44] Suppose that, at the time $t = 0$, the bacterial concentration is x_0. Let $x(t)$ denote the concentration at the time t.

It is assumed that the controlling quantities are not affected by the bacterial growth and metabolism. Therefore, of course, we do not model them. If an $x(t)$ function describes the time variation of the concentration of a population then the instantaneous *specific* growth rate, $\mu(t)$, at the time t is defined by

$$\mu(t) = \frac{\frac{dx(t)}{dt}}{x(t)} \tag{1}$$

In other words, the specific growth rate is the rate of increase *per* the population density that has produced that increase. The pure exponential growth model assumes that the specific growth rate of the population is constant:

$$\frac{dx(t)}{dt} = \mu x(t) \tag{2}$$

Introduce the notation

$$y(t) = \text{Ln}\, x(t) \tag{3}$$

$$y_0 = y(0) = \text{Ln}\, x(0) = \text{Ln}\, x_0 \tag{4}$$

By means of this, the differential equation (1) can be written as

$$\frac{dy(t)}{dt} = \frac{d(\text{Ln}\, x(t))}{dt} = \frac{\frac{dx(t)}{dt}}{x(t)} = \mu \tag{5}$$

Therefore, while the instantaneous *growth rate* can be represented by the slope of the "$x(t)$ vs. t" curve at the time t, the *specific growth rate* can be measured by the slope of the "Ln $x(t)$ vs. t" curve (Figure 16-1A and B). In practice, however, it is more usual to plot the 10-based logarithm of the concentration, log $x(t)$ against time. In this case, the slope of the "log $x(t)$ vs. t" curve is Ln 10 = 2.3-times less than μ, the "real" specific growth rate.

The solution for the differential equation (1), at the given initial value, is the following:

$$x(t) = x_0 e^{\mu t} \tag{6}$$

or, expressed by the natural logarithm of the cell concentration as follows:

$$\text{Ln}\, x(t) = y(t) = y_0 + \mu t = \text{Ln}\, x_0 + \mu t \tag{7}$$

Again, the usual log $x(t)$ concentration can be obtained by using the conversion factor Ln 10 = 2.3, keeping in mind that this is the factor between the natural and the 10-based logarithm:

$$\log x(t) = \log x_0 + at \tag{8}$$

where $a = \mu/2.3$. This is in accord with what has been mentioned, that the slope of the growth curve on the $\log_{10}$ scale, a, differs from the specific growth rate by the factor 2.3.

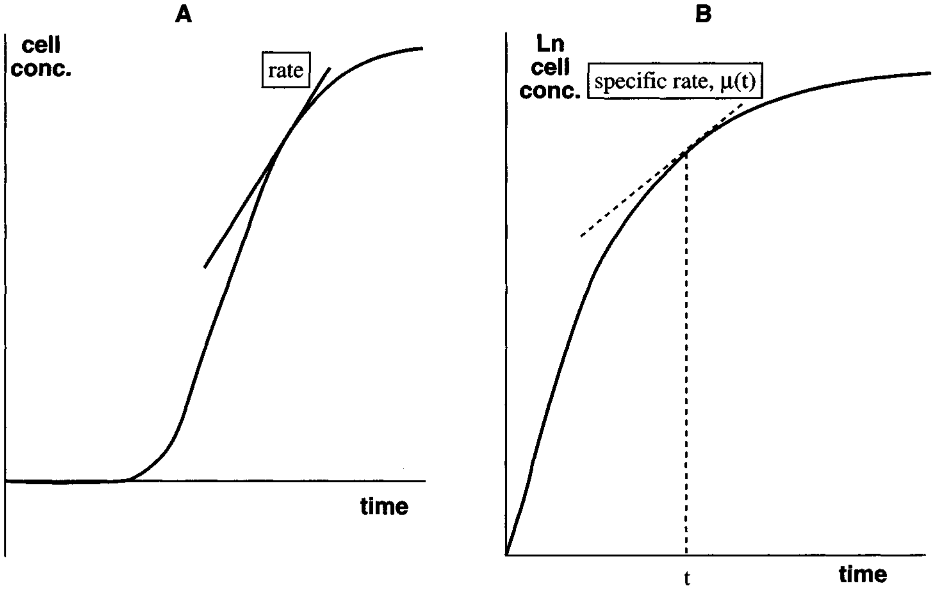
One can ask, what time interval is needed for the population to double? From the equation (6), simple calculation shows that if

$$t_D = \frac{\text{Ln}\, 2}{\mu} \approx \frac{0.69}{\mu} \tag{9}$$

then

$$x(t + t_D) = x_0 e^{\mu(t+t_D)} = e^{\mu t_D} x_0 e^{\mu t} = 2x(t) \tag{10}$$

The rate is measured on linear scale, while the specific rate on (natural) log scale.



If the cell concentration was modeled by a classical sigmoid function (in this case Gompertz) then, on the log scale, lag is not apparent.

Figure 16-1 The Difference between Growth Rate and Specific Growth Rate Using the Same Dataset.

Therefore, the doubling time is constant for exponential growth, and it can be calculated from the specific growth rate by means of formula (9). From a, the slope of the "log $x(t)$ vs. t" curve, the doubling time can be calculated as

$$t_D = \frac{\log_{10} 2}{a} \approx \frac{0.3}{a} \tag{11}$$

The doubling time, t_D for a population is not necessarily the same as the mean of the generation times of the individual cells, t_G.[73] They are equal only if the cells divide synchronously. If they do not grow synchronously, then the average cell cycle time of the individual cells is somewhat higher than the time necessary to double the whole population. For example, if the dynamics of the population can be closely related to the so-called Poissonian random birth process, which is widely observed in nature,[29] then the mean generation time of individual cells will be close to $t_G = 1/\mu$, which is almost 40% higher than $0.69/\mu$, the doubling time of the whole population.

16.4.3 Transition from Exponential to Stationary Phase

Let $x_{\max}$ be the maximum population concentration and let

$$y_{\max} = \text{Ln } x_{\max} \tag{12}$$

Suppose that the growth rate follows the model of Richards[61]:

$$\frac{dx(t)}{dt} = \mu_{\max} x(t)\left(1 - \left(\frac{x(t)}{x_{\max}}\right)^m\right) \tag{13}$$

Here, $\mu_{\max}$ is a model constant that can be called the (potential) maximum specific growth rate because the smaller the population concentration, ie, in the early exponential phase, the closer the specific growth rate is to $\mu_{\max}$. This is because, using the definition (3), we obtain the following:

$$\mu(x) = \frac{\frac{dx(t)}{dt}}{x(t)} = \mu_{\max}\left(1 - \left(\frac{x(t)}{x_{\max}}\right)^m\right) \tag{14}$$

However, as long as the actual cell concentration $x(t)$ is much smaller than its maximum, $x_{\max}$, the instantaneous specific growth rate is close to $\mu_{\max}$.

We express the solution of the differential equation (13) in terms of $y(t)$, the natural logarithm of the cell concentration:

$$y(t) = y_0 + \mu_{\max} t - \frac{1}{m}\text{Ln}\left(1 + \frac{e^{m\mu_{\max} t} - 1}{e^{m(y_{\max} - y_0)}}\right) \tag{15}$$

In the most frequently used form, the parameter m is taken to be 1, in which case the logistic function can be obtained for the bacterial concentration (not for its logarithm!). Indeed, expressing the above formula in terms of $x(t)$, at $m = 1$, one can obtain the following:

$$x(t) = \frac{x_{\max}}{1 + \left(\frac{x_{\max}}{x_0} - 1\right)e^{-\mu_{\max} t}} \tag{16}$$

This is the formula of the logistic function.

The model (14) states that the instantaneous specific growth rate of the population, $\mu(x)$, at the moment when the population concentration is x, is practically $\mu_{\max}$, until the population density becomes comparable to its maximum, $x_{\max}$. In other words

$$\frac{\frac{dx(t)}{dt}}{x(t)} = \mu(x) = \mu_{\max} u(x) \tag{17}$$

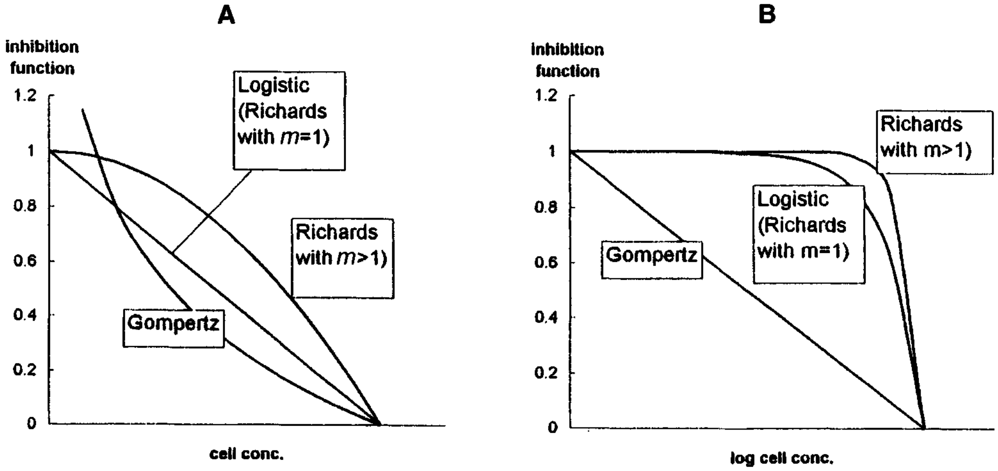
where $u(x)$ will be called an *inhibition* function. As the population concentration increases, the inhibition is stronger and stronger, ie, $u(x)$ is monotone decreasing and the population is less and less able to increase (Figure 16-2A and B). This structure is typical of all the models describing the transition to the stationary phase. The well-known models of Gompertz, Monod, etc, differ mainly in the way in which their inhibition function is derived.

Monod[53] modeled $u(x)$ with a Michaelis-Menten–type inhibition caused by the substrate concentration as shown here:

$$u(x) = \frac{S}{S + K_s}, \quad \frac{dx}{ds} = \frac{1}{-Y} \tag{18}$$

where K_s and Y are constant. As Baranyi and Roberts[5] pointed out, this model complies with the above definition of an inhibition function because it can be transformed into a model of the structure (17), with monotone decreasing inhibition function.

Another well-known model can be obtained by limiting the exponential growth as suggested by Gompertz.[34] Here, the limiting function is logarithmic:



The shape of the inhibition function depends on the scale of the axes.

Figure 16-2 Modeling the Transition from the Exponential into the Stationary Phase by the $u(x)$ Inhibition Function. **A**, The inhibition function against x, the cell concentration. **B**, The same inhibition function against $\log x$, the logarithm of the cell concentration.

$$\mu(x) = \frac{\frac{dx(t)}{dt}}{x(t)} = c\mathrm{Ln}\left(\frac{x_{\max}}{x(t)}\right) \qquad (19)$$

If smaller and smaller x cell concentrations are considered, then the specific growth rate, $\mu(x)$, converges to infinity. The model constant c is certainly not the (potential) maximum specific growth rate, which quantity does not even exist (Figure 16-2A). The main difference between the shapes of the Richards and Gompertz inhibition function is that the former has a value between 0 and 1, while the latter has no upper boundary. Therefore, the Gompertz model is not suitable, for example, to extrapolate the specific growth rate (or generation time) of the cells when the concentration was lower than x_0.

Further details on similar models can be found in Rubinow[73] and Turner et al.[82] It is important to see that Richards' family of functions (including the logistic), as well as the Gompertz function, is a sigmoid curve only for $x(t)$, the bacterial concentration, but not for its logarithm, $y(t)$ (see Figure 16-1A and B). The typical representation of a bacterial batch culture, however, is to plot the logarithm of the cell concentration against time and, in most cases, the result is a sigmoid curve. A possible empirical curve-fitting procedure is to fit sigmoid functions, augmented with an additive term, to these data. This approach, especially with the Gompertz function, was followed by many authors in recent years in predictive microbiology.[17,32,90] The Gompertz model has deep roots in population dynamics[37] but if the Gompertz function is "modified" to apply to the logarithm of the population size, then it should not be referred to as the *Gompertz model.* The terms function and model should be differentiated.[5] The phrase "Gompertz function fitted to the logarithm of the bacterial concentration" is correct, but the same phrase with "Gompertz model" is not. The differential-equation-form[84] is another form of the same, "modified," Gompertz model.

16.4.4 Transition from Lag to Exponential Phase

The classical growth models (Gompertz, logistic, etc) applied to the cell concentration do not show sigmoid characteristics on the log scale (Figure 16-1B). Therefore, they are not suitable for modeling the lag phase as traditionally defined, ie, the intercept of the tangent to the inflexion with the initial concentration. According to that (Figure 16-3), the end of the lag phase is the intercept of the lower asymptote and the tangent drawn to the inflection point of the bacterial curve plotted on the log scale.[56]

The problems with modeling the lag originate from this geometrical definition. Lag should be interpreted only if there is a distinct time when the environment changes suddenly (such as at inoculation), which can be considered the beginning of the lag. This means that the so-called autonomous structure of the equation (17) (namely, that the in-

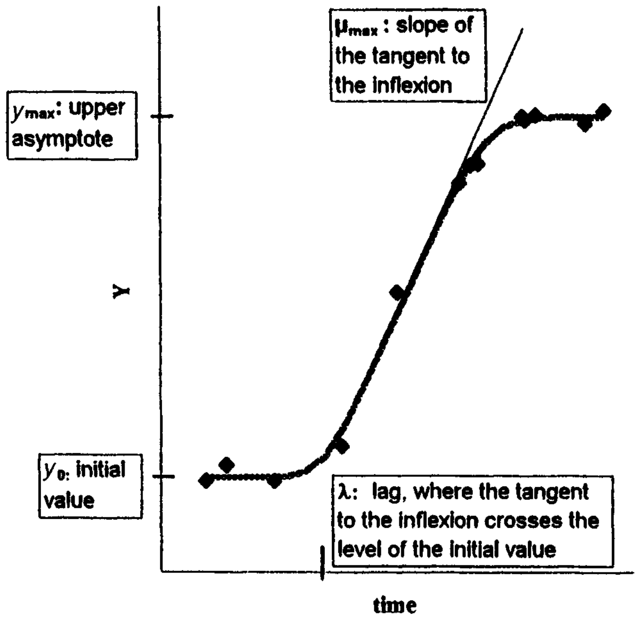


Figure 16-3 Bacterial Growth Curve Fitted by a Four-Parameter Sigmoid Curve.

stantaneous specific rate depends only on cell concentration) is not suitable to model lag phase.

For an individual cell, the lag is a waiting time spanning from the sudden change in the environment to the first division. At the population level, this waiting time has a distribution. The relationship between the distribution of the lag and the above geometrical definition is not at all straightforward.

Put the equation (17) in a different context. After rearranging

$$\frac{dx(t)}{x(t)} = \mu(x)dt$$

The left-hand side of this equation is an infinitesimal form of the probability that a randomly chosen cell divides in the $[t, t + \Delta t]$ interval. A probabilistic lag concept can be introduced as follows.

Multiply the right-hand side by the factor $\alpha(t)$, which expresses the probability that, at the time t, a randomly chosen cell is a daughter cell (ie, it is not from the initial generation). The proportion of these cells converges to 100% with time, therefore the values of $\alpha(t)$ is between 0 and 1 and monotone increasingly converges to 1 as t converges to infinity. The obtained relation reads as follows: *prob*(division happens in $[t, t + \Delta t]$) = $\alpha(t)\,\mu(x)\,\Delta t$.

Translating this model back to the population level:

$$\frac{\frac{dx(t)}{dt}}{x(t)} = \alpha(t)\mu(x)u(x) \qquad (20)$$

The probability distribution function $\alpha(t)$ is equivalent to the concept of *adjustment function* as defined by Baranyi and Roberts.[4] The $\alpha(0)$ initial value measures the proportion of the cells that are ready for the new environment.

The value $\alpha(t)$ determines not only the value of lag (λ, in what follows) but also affects the curvature from the lag to the exponential phase. For example, if

$$\alpha(t) = \begin{cases} 0 \text{ for } t < \lambda \\ 1 \text{ for } t > \lambda \end{cases}$$

then the curvature is a breakpoint, ie, the model of intercepting straight lines can be obtained.

Because there are no reliable measurements on the nature of this $\alpha(t)$ distribution function, some idealistic and empirical simplifications have to be introduced. Baranyi and Roberts[4] introduced a two-parameter adjustment function (which, as has been pointed out, is the same as the $\alpha(t)$ distribution function for the lag) based on the hypothesis that there is a rate-limiting intracellular bottleneck substance, $P(t)$, which must be built up during the lag. That substance is assumed to inhibit the growth according to a Michaelis-Menten kinetics, as follows:

$$\frac{\frac{dx(t)}{dt}}{x(t)} = \frac{P(t)}{K_P + P(t)}\mu_{\max}u(x) \qquad (21)$$

If, during the lag, that critical substance is exponentially growing, at a constant v specific rate, then the growth of the cell population can be described by

$$\frac{\frac{dx(t)}{dt}}{x(t)} = \frac{q_0 e^{vt}}{1 + q_0 e^{vt}}\mu_{\max}u(x) \qquad (22)$$

where $q_0 = P_0/K_P$. Therefore, the wanted distribution function can be written as

$$\alpha(t) = \frac{q_0}{q_0 + e^{-vt}} \qquad (23)$$

In this model, q_0, the ratio between P_0 (the initial value of the critical substance) and K_P (the Michaelis-

Menten constant) summarizes the "readiness" of the cells for the new environment. The parameter $\alpha(0) = \alpha_0 = q_0/(1 + q_0)$ is another measure of this readiness.

As can be proved mathematically, the mentioned geometrical measure of the length of the lag phase can be estimated by $\lambda = \exp(-\alpha_0)/v$.

Referring to the original paper for technical details, the adjustment model of Baranyi and Roberts[4] combined with Richards' growth model (see above) gives a differential equation, which can be solved explicitly:

$$y(t) = y_0 + \mu_{\max}A(t) - \frac{1}{m}\mathrm{Ln}\left(1 + \frac{e^{m\mu_{\max}A(t)} - 1}{e^{m(y_{\max} - y_0)}}\right) \quad (24)$$

where $A(t)$, the integral of $\alpha(t)$, can be considered as a gradually delayed measure of time:

$$A(t) = t - \lambda + \frac{\mathrm{Ln}\left(1 - e^{-vt} + e^{-v(t-\lambda)}\right)}{v} \quad (25)$$

16.4.5 Model Implementation

It is not easy, if at all possible, to fit the differential equation (22) to actual data using ordinary regression packages. This is why it is important to know whether the solution of a differential equation is expressible by a composition of elementary functions. If it is, then the solution is called explicit and it can be readily used in ordinary software packages, such as spreadsheets, for prediction. Such an expressible solution, however, is never guaranteed and, in fact, more often than not a randomly chosen differential equation will not have an explicit solution. In our case, it does exist, as described by the equations (24) and (25).

Another important difference between the model (22) and its explicit expression (24) and (25) is their applicability for time-dependent conditions. Baranyi et al.[6] demonstrated that the model (22) is also valid under (not severely) changing environmental conditions. Simply, the environment dependence of the maximum specific rate must be incorporated in the model: $\mu_{\max} = \mu_{\max}(E(t))$, where $E(t)$ is the environmental profile. However, the explicit solution (24) will not be valid anymore and, commonly, the model can be simulated only approximately on a computer. The need for predictions in dynamically changing environments makes it inevitable that growth models should be derived in their differential equation form rather than in an explicit form.

When using an explicit model for numerical purposes, like simulation or curve fitting, it is sometimes advisable to reparameterize the original model. It is possible that a different parameterization of a model is more advantageous for computation than the parameterization used to develop the model.[58] For a simple example, consider the expression exp(1000) • exp(–1000). When typing this into the computer, the result should be obviously 1. However, most packages would give overflow error when carrying out this statement. But if we rearrange the formula and type exp(1000–1000), there is no problem with the exact answer.

Similar rearrangement is necessary when using the formulae (24) and (25) for fitting, otherwise the numerical instability can result in error messages. Baranyi and Roberts[4] reduced the number of the parameters, specifically for bacterial counts, in the following way. They assumed that the accumulation of the bottleneck substance, $P(t)$, during the lag, happens at the same specific rate that will be later the maximum specific rate of the population ($v = \mu_{\max}$) and, introducing $n = v/\mu_{\max}$, they fixed the value of n as $n = 1$ and the value of m as $m = 1$ (logistic limitation at the transition to the stationary phase).

In order to fit data other than bacterial counts that show sigmoidal behavior, it is useful to reparameterize the equations (24) and (25) in such a way that the optimal curvature parameters do not depend on the scale of the data. For this reason, introduce the parameter $h_0 = v$ and, in return, drop the parameter v from the model. The value h_0 will be a dimensionless curvature parameter characterizing the transition from the lag to the exponential phase. The significance of this reparameterization is that the h_0 does not depend on the time scale used, and the shape of the transition from the lower asymptote to the linear phase remains the same after using, say, hours instead of days.

For the same reasons, it is worth reparameterizing the other curvature parameter, m, and using $M = m(y_{\max} - y_0)$ instead. The value M will describe the transition from the exponential to the stationary phase. The higher its value, the closer is the transition to a real "breakpoint" and the curvature does not depend on the used scale for the y axis (except, of course, for nonlinear transformations of that axis).

By fixing the h_0 and M curvature parameters, four parameters remain to be fitted: y_0, $y_{\max}$, $\mu_{\max}$, and λ. Fitting this model is comparable to the fitting of the classical growth functions like logistic or Gompertz, augmented by an additive term.[90]

Note that the parameter $\mu_{\max}$ is not exactly the steepest slope of the actual growth curve, but expresses a "potential" maximum specific growth rate that is characteristic to the population only if the initial concentration level is not very high. The difference between the steepest slope and μ_{max} is noticeable if the h_0 and M curvature parameters are small and/or y_0 is close to y_{max}.

The reparameterized form of the equations (24) and (25), with dimensionless h_0 and M curvature parameters, can be written as follows:

$$y(t) = y_0 + \mu_{max} A(t) - \frac{y_{max} - y_0}{M} \bullet \mathrm{Ln}\left(1 - e^{-M} + e^{-M} \frac{y_{max} - y_0 - \mu_{max} A(t)}{y_{max} - y_0}\right) \quad (26)$$

where

$$A(t) = t - \lambda\left(1 - \frac{1}{h_0}\mathrm{Ln}\left(1 - e^{-h_0 \frac{t}{\lambda}} + e^{-h_0\left(\frac{t}{\lambda} - 1\right)}\right)\right) \quad (27)$$

The equations (26) and (27) describe a (generally sigmoid) function with six parameters, two of which, h_0 and M, are curvature parameters that are not easy to fit and it is advised to keep them as fixed numbers. In our experience, $h_0 = 10$, $M=10$ are good empirical constants for them, for not very sharp curvatures.

Below, that BASIC subroutine is listed, which is used in an in-house program in the Institute of Food Research, UK, to fit sigmoid curves. The routine can be used, for example, in combination with the Levenberg-Marquardt method of the Numerical Recipes.[57,77] The routine returns with the function value y for the following input parameters:

`t:`	time
`mu, lag, y0, ymax:`	parameters μ, λ, y_0, y_{max} to be optimized
`M, h0:`	fixed curvature parameters
`M = 0:`	a symbolic value for y_{max} = infinitely large (upper asymptote does not exist)
`h0= 0:`	a symbolic value for the case when there is no lag

The routine also calculates the derivatives

$$\frac{dy}{d\mu}, \frac{dy}{d\lambda}, \frac{dy}{dy_0}, \frac{dy}{dy_{max}}$$

and stores them in the fields `dyda(1)...dyda(4)`.

Remember that `Log` is meant in BASIC as natural logarithm.

```
BASIC CODE:

largeNumber=100: upBreak=20
bt = 1
If h0 = 0 Then
   At = t: dApdlag = 0
ElseIf h0 >= upBreak Then
   If t < lag Then
      At = 0: dApdlag = 0
   Else
      At = t - lag: dApdlag = -1
   End If
ElseIf h0 > 0 Then
   tpl = Abs(t / lag)
   If -h0 * tpl + h0 > largeNumber Then
      At = 0: dApdlag = 0
   Else
      bt = 1 - Exp(-h0 * tpl) + Exp(-h0 * tpl + h0)
      If bt <= 0 Then errCode = 3: Exit Sub
      At = t - lag + lag * Log(bt) / h0
      If At < 0 Then At = 0
      dApdlag = tpl * (bt - 1) / bt + Log(bt) / h0 - 1
   End If
Else
   At = t - lag * (1 - Exp(h0 * t))
   If At < 0 Then At = 0
   dApdlag = 1 - Exp(h0 * t)
End If
yNoEnd = y0 + mu * At
If M = 0 Then
   y = yNoEnd: dyPdmuA = 1
   dyda(1) = At: dyda(3) = 1
ElseIf M >= upBreak Then
   If yNoEnd >= ymax Then
      y = ymax: dyPdmuA = 0: dyda(1) = 0: dyda(3) = 0
   Else
      y = yNoEnd: dyPdmuA = 1: dyda(1) = At: dyda(3) = 1
   End If
Else
   cm = (ymax - y0) / M: mupcm = M * mu / (ymax - y0)
   If -M + mupcm * At > largeNumber Then
      y = ymax: dyPdmuA = 0: dyda(3) = 0
   Else
      Mt = Exp(-M + mupcm * At)
      Et = 1 - Exp(-M) + Mt
      dyPdmuA = 1 - Mt / Et
      y = y0 + mu * At - cm * Log(Et)
      dyda(3) = 1 + (Log(Et) - Mt / Et * mupcm * At) / M
   End If
End If
dyda(1) = dyPdmuA * At
dyda(2) = dyPdmuA * mu * dApdlag
dyda(4) = 1 - dyda(3)
Exit Sub
```

16.4.6 Accuracy of Predictive Models

As has been mentioned, the basic requirement is to predict the bacterial concentration, $x(t)$ for any time $t > 0$. For this, the variation of the main growth parameters with environmental factors such as temperature, water activity, etc, is modeled. The type of models used is extensively discussed in McMeekin et al.[49] Here we consider a practical concern: the accuracy of those models.

It is assumed that the potential maximum specific growth rate of the population achieved in a constant environment, as explained in the previous points, is an intrinsic growth parameter (independent of the experimenter and of the history of the cells). Of course, due to the inevitable biological complexity, replicate experiments show a certain variance. Under favorable conditions, the estimated standard deviation of the maximum specific growth rate of replicated *Listeria monocytogenes* growth curves using viable counts generated in the laboratory was about 7% to 10% of the average estimated value.[5] In accord with what follows, we say that the method of measuring the specific growth rate by the above way has about 10% error. Another possible terminology is to say that the method has an accuracy factor of about $p = 1.1$. This latter terminology was used by Ross[70] who defined the "accuracy factor" of a model as the antilog value of the average difference between the log(observed) and log(predicted) values of the modeled responses (generally specific rates or doubling times). The only modification we suggest to this definition is that the interpretation of the "average difference" should be not arithmetical average but square-root-average, consistently with the least squares principle of statistics. Therefore, let the "amended accuracy factor," p, be defined as

$$\log p = \sqrt{\frac{\sum\left(\log(observed) - \log(predicted)\right)^2}{n}}$$

where n is the number of observations and the summation is meant through these observations.

The advantage of this definition is as follows. When modeling the variation of the specific growth rate with the environmental factors like temperature, water activity, etc, the logarithm function is commonly used as a variance-damping link function. Therefore, if the standard error of fitting for this model is denoted by SE, then $\log p$ will be exactly SE. Note that if the natural logarithm of the responses were modeled then, of course, $\text{Ln}\, p = \text{SE}$.

In our experience, the amended accuracy factors of most predictive models for the specific growth rate (or doubling time) of viable count cultures are between 1.2 and 1.5, depending on the number of environmental factors in the model and on the range of those factors. That means that 10% to 40% additional error was generated compared with the 10% error resulting from calculation of the rate from a viable count growth curve. However, even with this additional error, the predictive models are extremely useful in food microbiology.

16.5 PROSPECTS

Models should be regarded as useful tools to assist decision making rather than substitutes for experience and knowledge of microbial responses. Users of models should be conversant with the manner in which the models have been produced, and their relevance to microbial responses in foods. Ideally, users should "validate" models themselves by limited challenge tests, or by comparison with historical data of microbial responses in the food commodities of particular interest.

Experience indicates that models of microbial growth developed in laboratory media and including no more than four controlling factors, usually pH, % brine (or a_w), temperature, and one other factor, generally offer a rapid, convenient, and reasonable "first estimate" of how foodborne bacterial pathogens will behave in many food systems. As larger quantities of data for a broader range of microbes covering more and more controlling factors (CO_2, nitrite, ethanol, organic acids, naturally occurring antimicrobials, etc) are accumulated, confidence that modeling is the most cost-effective way forward is likely to increase. This modeling approach has been applied successfully to bacteria, yeasts,[40] and molds.[33]

The relevance of models to growth of pathogens in foods should be checked carefully before predictions are used in commercial circumstances or for regulatory purposes. The least costly means of such checking is by comparison with independent data in the literature. To avoid costly and time-wasting duplication of effort, comparisons of model predictions and independent estimates of microbial responses should be accumulated (eg, in a database) to identify those circumstances where models can be relied upon, and those circumstances where they fail and, therefore, should not be used or should be further investigated. The accumulated database of comparisons should include all the common food commodities, and the processes applied to them (raw, cooked, fermented, dried, cured, etc). Currently, no institution or organization has come forward to coordinate and support a centralized database, and comparisons of models with values collected from the literature rests with those producing or using models.

Existing validated models give reasonable predictions of growth rates, but less valid estimates of lag times. This is understandable because the growth rate depends upon the environment in which growth is occurring and will increase to the maximum permitted by that environment (as determined by pH, a_w, and temperature). In contrast, the lag time is dependent on the prior history of the cells comprising the inoculum, and that condition has not been researched in sufficient detail to be embodied in current models. For instance, dramatic fluctuations in temperature are known to induce lag, but the quantitative relationship between the magnitude and rates of temperature fluctuation with time and the duration of lag induced have not been quantified. It is an important topic for research if models are to be more usefully employed in processing environments. Similarly, the effect on lag of changes in pH and a_w remain to be explored.

If there was general agreement on indices of model performance, comparisons of models would be facilitated.[70] As a general rule, models should be as simple as reasonably possible with the minimum number of coefficients. This so-called *parsimony* is vital when using models for predictions. The goodness of fit, how a model fits to observations, can, in principle, be improved infinitely by just adding more parameters to the model. A special case of this overparameterization is the application of third-order polynomials for growth rate models, as criticized in Baranyi et al.[7] Importantly, the more overparameterized the model, the greater the chance that it gives nonsense predictions, especially in regions where data are scarce. Predictions should always make "biological sense," and reflect the experience of microbiologists of the growth and death responses of organisms in foods.

While the potential consequences of overparameterization have been addressed, as indicated above, agreement on the definition of the usable domain of models is still under active discussion.[7] It has been proposed that this domain can be sensibly proscribed by, for example, the minimum convex polyhedron (convex hull) around the conditions under which data have been generated.[7] Extrapolation of the ranges of conditions to give predictions beyond the multidimensional space in which data that led to the model were accumulated decreases enormously the reliability of predictions, and to an extent that is impossible to quantify.

Kinetic and probabilistic models are already being combined[60] leading to estimates of both the rate of growth and the likelihood of it being achieved.

There are opportunities to advance predictive microbiology by applying instrumental methods, eg, impedance,[25] conductance,[41] indirect conductance,[24] turbidity,[22] gas chromatography,[8] gradient plates coupled with image capture and analysis software,[81] and gel cassette coupled with laser scanning.[13] However, it must be kept in mind that the measured responses using these techniques are not necessarily linearly proportional to the size (numbers) of the bacterial population, and, therefore, these models may not be comparable to models based on viable counts. Nevertheless, automated measurements can be useful to establish certain characteristic features of predictive models, such as growth–no growth boundaries, optimal values of environmental values, interactions between factors, etc.

Most currently available growth models would be improved, and more generally useful, if their ranges of conditions were greater, in particular covering the extreme ranges of conditions permitting growth.

Once a model has been validated for a particular food group, eg, meat products, so that predictions of growth closely mimic reports in the literature for the same environmental conditions, the models become useful guides to bacterial growth in those foods. Confidence intervals can be added to predictions,[46] but are often large, especially toward the extreme conditions of growth. Because lag time is a function of the prior history of the inoculum and of changes in environmental conditions, eg, changing temperature, models commonly overestimate the overall growth response. Although this is regarded as "failing safe," in that the estimate is a "worst case scenario," the value of models could be improved greatly by research effort being directed at understanding the quantitative relationship between changing conditions and the lag time subsequent to those changes. As an example, when temperature changes within the range of temperatures for growth, growth rate adjusts almost instantaneously to the new temperature. However, if the temperature exceeds the maximum for growth or falls below the minimum for growth, or even approaches those values, some additional lag is induced, and that is not currently taken into account in models.[6] New experimental approaches are required to correlate those changes with the growth response. Despite such limitations, models of thermal death and growth of pathogens are already proving useful in quantitative risk assessments.[88]

Whatever the experimental approach to developing models and comparing model outputs with values from the literature or with those from challenge tests, well-organized databases are essential. Commonly, the experimental data are collected and initially stored without critical evaluation ("database 1"). Critical examination commonly rejects part of the collected raw data, leading to "database 2." All, or part, of "database 2" may be used to develop a preliminary model, which often identifies the need for additional experimentation to consolidate or extend the model. The dataset, or subset of data, used to develop a model must be readily identifiable with it. Subsequently, even more data may be added to improve the model further. Each step needs careful documentation if datasets leading to intermediate and final models are to be retrievable.

Similarly, data compiled from the literature require careful supporting documentation if optimal use is to be made of them. Every measure of growth or death (specific growth rate, generation time, time to turbidity, D-value, etc.) must be supported by details of the experimental environment including the following: pH and the acid used, % brine or a_w and the humectant, temperature(s) and duration of the experiment, other preservative factors and their concentrations, gas atmosphere, the size of the inoculum, methods to recover the microbe(s), and any process that might affect the estimate, such as sudden change of temperature or pH from the subculture to the test conditions and any other changes that could lead to "injury" and lag being induced.

Predictive models began via purely empirical approaches, supported by statistical tools. Today, more and more mechanistic elements are used in their construction, in parallel to more and more advanced use of mathematical modeling and computational tools. The key to this development is the cooperation between microbiologists and food technologists on the one hand and mathematicians and statisticians on the other. In this chapter, some examples have been given to demonstrate this process. This can lead not only to better understanding of bacterial kinetics but also to more accurate predictions and, ultimately, safer and better quality food.

If "predictive microbiology" can be realized widely for thermal death, nonthermal death (survival), and growth conditions, food microbiology will advance immeasurably. Once a model has been validated in a product, it can be used at all stages of the food chain from harvesting, through production and processing, to distribution and retail sale. Modeling is already being incorporated into hazard analysis critical control point (HACCP) systems, product development, and quantitative risk assessment. Coupled with process engineering and knowledge of thermal properties of foods, predictive modeling will prove an increasingly powerful tool in the quest to optimize quality and safety.[74,91]

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II Microbial Ecology of Different Types of Food

17 Fresh Red Meats

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17.1 INTRODUCTION

Approximately 121 million metric tons of meat were produced by selected countries of the world during 1995.[122] China produced the greatest amount, 37 million metric tons. The United States (US) produced the second largest amount, 19 million metric tons, and beef and veal accounted for about 60% of the meat produced. In China the production focused on pork.

China and the US were forecasted to export 358 and 1,367 thousand metric tons of meat in 1997, respectively.[128] China was forecasted to import 3,000 metric tons of red meat, limited to lamb/mutton/goat, whereas the US was forecasted to import 1.3 million metric tons of all red meats. The largest exporters of red meats are Australia and the US with a total of 1.37 and 1.18 million metric tons in 1995, respectively, and 1.39 and 1.37 million metric tons for 1997, respectively. The largest importers of red meats are Japan, the US, and the Russian Federation with imports of 1.84, 1.28, and 1.08 million metric tons in 1995, respectively, and imports of 1.87, 1.34, and 1.27 million metric tons in 1997, respectively. Historically, meat has been shipped frozen but some of the newer technologies of thorough cleansing of carcasses, strict temperature control, and vacuum and modified atmosphere packaging have allowed for shipping of chilled, fresh meat, even in international trade.

The steps involved in the conversion of animals into meat (Figure 17-1)[129] include transporting the live animal from the production unit to the slaughter facility, then killing, skinning, eviscerating, washing, chilling, cutting, boning, and packaging. Pigs are not usually skinned so the steps differ in that the carcasses are scalded, dehaired, singed, and polished before evisceration, chilling, cutting, boning, and packaging (Figure 17-2). Live animals and the resultant carcasses are inspected for wholesomeness during this process. Wholesomeness means the meat resulted from healthy animals but does not imply freedom from spoilage nor potentially pathogenic bacteria.

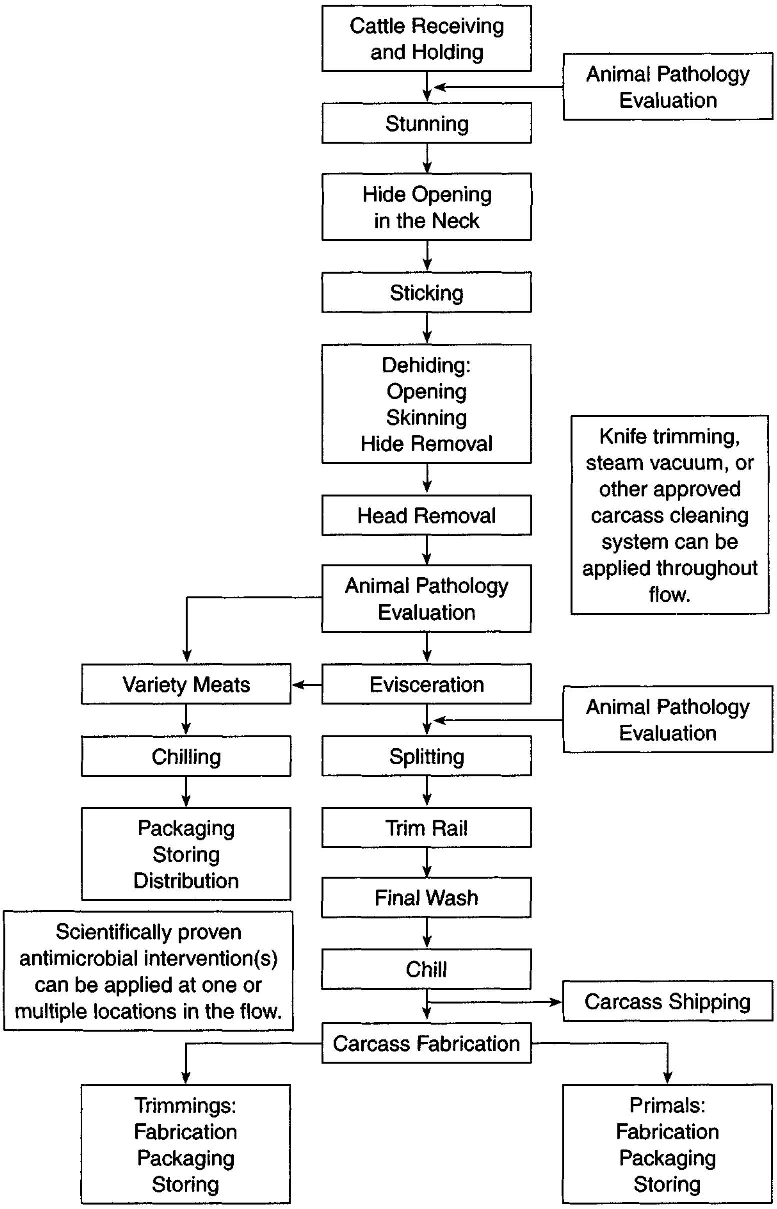
Cattle Receiving and Holding
Animal Pathology Evaluation
Stunning
Hide Opening in the Neck
Sticking
Dehiding: Opening Skinning Hide Removal
Knife trimming, steam vacuum, or other approved carcass cleaning system can be applied throughout flow.
Head Removal
Animal Pathology Evaluation
Variety Meats
Evisceration
Animal Pathology Evaluation
Chilling
Splitting
Packaging Storing Distribution
Trim Rail
Final Wash
Scientifically proven antimicrobial intervention(s) can be applied at one or multiple locations in the flow.
Chill
Carcass Shipping
Carcass Fabrication
Trimmings: Fabrication Packaging Storing
Primals: Fabrication Packaging Storing

Figure 17-1 Flow Chart of Beef Slaughter, Fabrication, and Packaging. *Source:* Reprinted from *Guidebook for the Preparation of HACCP Plans,* 1997, Food Safety and Inspection Service, U.S. Department of Agriculture.

Red meats that have been processed and cooked properly provide the essential amino acids, minerals, vitamins, and other nutrients for human nutrition. Microbial contamination of fresh red meat occurs mainly on the surface. The internal portions of the intact meat are usually free of spoilage or pathogenic microorganisms unless the slaughter animal had a septicemic condition or had been infected with parasites. Viruses associated with meat from slaughter animals, to date, have not posed a serious concern to the health of the consumer. Although molds and yeasts may be present, they are of less concern than the presence of bacteria; for prions see Chapter 57.

Both spoilage and potentially pathogenic bacteria are present on the surface of the slaughter animal and in the intestinal tract at the time of slaughter. Facilities and red meat become contaminated from these two sources during slaughter and processing. Personnel can be another source of contamination and cross-contamination. Additional contamination and/or microbial growth may occur during storage, distribution, and handling.

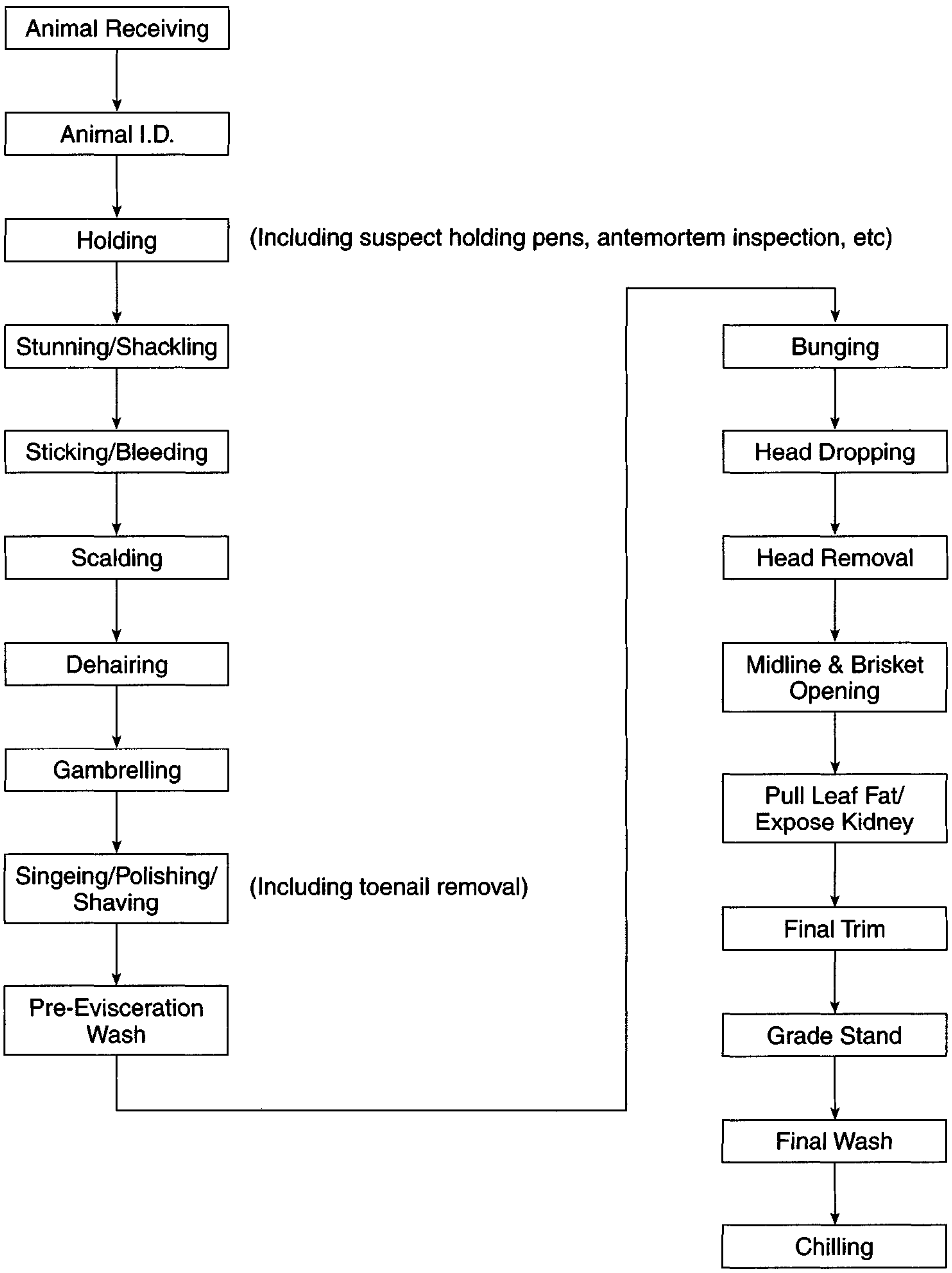

Figure 17-2 Flow Chart of Pork Slaughter (skin on carcass). *Source:* Reprinted with permission from Developing and Implementing HACCP in Small and Medium-Sized Plants—Workshop, American Association of Meat Processors, American Meat Science Association, and National Association of Meat Purveyors, © 1998, American Association of Meat Processors.

Initially, the surface microflora of red meat consist of a mixture of the microbes. If conditions are appropriate, both spoilage and pathogenic microorganisms may multiply on the surface. Measures taken by the food industry to reduce the number of microorganisms present on raw meat include the following: (1) procedures to reduce the initial surface contamination of carcasses, (2) prevention of cross-contamination during processing, (3) the use of methods for decontamination (cleansing) of meat, and (4) preservation and thermal processing.

Parasites can also be a serious problem. Antemortem and postmortem inspections, freezing, and cooking have been used for many years to prevent the transmission of parasites such as *Trichinella spiralis.* Less attention has been given to other significant parasites, such as *Toxoplasma gondii,* that may be present on pork, lamb, and other meats. If the meat is consumed without thorough cooking, these parasites may cause serious illness in humans, particularly in people who are immunosuppressed. In the healthy individual, *Toxoplasma gondii* can remain viable for years until health is challenged, at which time the parasite may reproduce and cause serious consequences, such as dementia. In females pregnant for the first time, the parasite can cause difficulties such as blindness or miscarriage of the fetus.

Extensive research effort has been expended on decontamination procedures to compensate for contamination that occurs during animal production, slaughter, evisceration, chilling, carcass separation, and packaging. Many of the proposed methods of decontamination are not totally effective (for example, the use of chlorinated washes, steam pasteurization, and ultraviolet treatment) or, as in the case of irradiation, are not generally acceptable to consumers. Greater attention is being focused, therefore, on methods to eliminate pathogenic microorganisms from carcasses, to reduce cross-contamination and extend the storage life of the meat.

17.2 COMPOSITION

Meat from slaughter animals consists of about 64% to 80% moisture, 16% to 20% protein (N × 6.25), 6% to 10% fat, and 1% ash (Table 17-1). The composition of the various muscles differs depending upon the muscle function and the proximity of adipose tissue, as well as animal species, breed, age, gender, and nutritional status. Younger animals typically have lower levels of deposited fat in their adipose sites. During maturation, fat is deposited within adipose tissue at a relatively rapid rate if adequate feed is available, whereas lean muscle is pro-

Table 17-1 Composition of Trimmed Raw Retail Cuts, Separable Lean (Amount in 100 grams, edible portion)[115–117]

	Beef[a]	*Pork*[b]	*Lamb*[c]	*Lamb*[d]	*Veal*	*Chevon*	*Horse*
Proximate:							
water (g)	70.62	72.34	73.42	73.78	75.91	75.84	72.63
energy (kcal)	144	143	134	128	112	109	133
(kj)	604	599	561	537	469	455	556
protein [N ×6.25] (g)	20.78	21.07	20.29	20.75	20.20	20.60	21.39
total lipid (g)	6.16	5.88	5.25	4.41	2.87	2.31	4.60
carbohydrate (g)	0	0	0	0	0	0	0
ash (g)	1.02	1.04	1.06	1.12	1.08	1.11	0.99
Minerals: (mg)							
calcium	6	16	10	8	15	13	6
iron	2.13	0.91	1.77	1.64	0.85	2.83	3.82
magnesium	22	23	26	19	25	—	24
phosphorus	199	211	189	202	211	180	221
potassium	356	380	280	171	328	385	360
sodium	63	57	66	46	86	82	53
zinc	4.33	2.08	4.06	3.01	3.23	4.00	2.90
copper	0.08	0.07	0.12	0.10	0.11	0.26	0.14
manganese	0.01	0.01	0.02	0.02	0.03	0.04	0.02
Vitamins:							
ascorbic acid (mg)	0	0.7	—	—	—	—	1.0
thiamine (mg)	0.11	0.97	0.13	0.15	0.08	0.11	0.13
riboflavin (mg)	0.18	0.27	0.23	0.39	0.28	0.49	0.10
niacin (mg)	3.59	4.83	6.00	6.54	7.83	3.75	4.60
pantothenic acid (mg)	0.36	0.79	0.70	0.49	1.37	—	—
vitamin B_6 (mg)	0.44	0.51	0.16	0.14	0.43	—	0.38
folacin (mg)	7	5	23	—	13	5	—
vitamin B_{12} (mg)	3.25	0.67	2.62	2.71	1.43	1.13	3.00
vitamin A (RE)	0	2	—	—	—	—	—
(IU)	0	6	—	—	—	—	—

continues

Table 17-1 Continued

	Beef[a]	*Pork*[b]	*Lamb*[c]	*Lamb*[d]	*Veal*	*Chevon*	*Horse*
Lipids:							
fatty acids (g)							
saturated total	2.32	2.03	1.88	1.88	0.86	0.71	1.44
10:0	0	0.01	0.01	0.01	0	0	—
12:0	0	0.01	0.01	0.01	0	0	0.01
14:0	0.17	0.07	0.14	0.09	0.04	0.03	0.17
16:0	1.40	1.29	1.02	0.90	0.49	0.33	1.08
18:0	0.73	0.64	0.62	0.73	0.30	0.33	0.16
monounsaturated total	2.61	2.66	2.11	1.69	0.92	1.03	1.16
16:1	0.23	0.19	0.16	0.08	0.09	0.04	0.31
18:1	2.36	2.42	0.91	1.60	0.82	0.94	1.30
20:1	0.01	0.04	—	0	—	—	—
polyunsaturated total	0.24	0.63	0.48	0.22	0.30	0.17	0.65
18:2	0.19	0.51	0.36	0.13	0.21	0.10	0.29
18:3	0.02	0.02	0.07	0.07	0.01	0.02	0.36
20:4	0.03	0.07	0.05	0.02	0.08	0.06	—
cholesterol (mg)	59	61	65	74	83	57	52
Amino acids: (g)							
tryptophan	0.23	0.27	0.24	0.24	0.20	0.31	0.26
threonine	0.91	0.96	0.87	0.89	0.88	0.98	0.96
isoleucine	0.93	0.99	0.98	1.00	1.00	1.04	1.01
leucine	1.64	1.69	1.58	1.61	1.61	1.72	1.70
lysine	1.73	1.90	1.79	1.83	1.66	1.53	1.82
methionine	0.53	0.56	0.52	0.53	0.47	0.55	0.47
cystine	0.23	0.27	0.24	0.25	0.23	0.24	0.30
phenylalanine	0.81	0.84	0.83	0.84	0.82	0.72	0.88
tyrosine	0.70	0.73	0.68	0.70	0.64	0.63	0.67
valine	1.01	1.14	1.10	1.12	1.12	1.10	1.11
arginine	1.31	1.31	1.21	1.23	1.19	1.51	1.40
histidine	0.71	0.84	0.64	0.66	0.73	0.43	0.82
alanine	1.25	1.23	1.22	1.25	1.20	—	1.23
aspartic acid	1.90	1.95	1.79	1.83	1.74	—	2.10
gluconic acid	3.12	3.30	2.94	3.01	3.20	—	3.12
glycine	1.13	1.00	0.94	1.01	1.04	—	1.03
proline	0.92	0.85	0.85	0.87	0.84	—	0.99
serine	0.80	0.87	0.76	0.77	0.76	—	0.82

[a] Beef—trimmed to 1/4" fat.
[b] Pork—leg, loin, shoulder.
[c] Lamb—domestic choice.
[d] Lamb—imported frozen.

duced at a slower rate so that the fat-to-lean ratio increases. Moisture is retained at a relatively constant rate within the lean muscles but the moisture level of adipose tissue decreases as fat is deposited. The amount of connective tissue differs among the various muscles, and during maturation the amount of cross-linking within the connective tissues increases. It is not known whether connective tissue or the degree of cross-linking have any effect on microbial growth, although they may provide a physical barrier to the availability of nutrients. However, microorganisms attach readily to all physical components of meat such as chicken skin, chicken muscle connective tissue,[12] pork skin,[10,11] lamb and beef muscle,[10,23] and beef fat.[23]

Females deposit more fat than males and neutered males deposit more fat than intact males. Breed also affects the fat-to-lean ratio of carcasses. Dairy and dual-purpose cattle, for example the Guernsey, Limousin, and Charolais, have a lower or intermediate level of deposited fat than beef breeds such as Hereford or Aberdeen Angus. Genetic selection of pigs is responsible for significant changes in the muscularity and therefore conformation of the carcass and a drastic reduction in the amount of fat in the resultant meat. This selection of the very lean hogs has resulted in a temperament change of the pig, with some exhibiting a defect described as the "porcine stress syndrome" (PSS). This disorder is important to the pork industry because pigs sometimes die when subjected to stress, such as encountered during transport. PSS pigs may yield carcasses and meat that is pale, soft, and exudative (PSE). The PSE condition results from a rapid reduction of the meat pH post-slaughter. The lowered pH causes the proteins to approach their isoelectric points, thereby decreasing water-holding capacity and resulting in exudative meat.

The conversion of muscle of slaughter animals to meat involves the loss of available oxygen to the muscle cells, the utilization of available ATP, and the contraction of muscle fibers resulting in shortened sarcomeres, and in rigor mortis. Concurrently, there is the anaerobic utilization of some of the available glycogen, the production of lactic acid, and the reduction of the muscle/meat pH. The resolution of rigor mortis, interpreted on a consumer level as relaxation of the contracted sarcomeres, is due to sarcomere rupture at the "Z" bands and the activation of cathepsins, lipases, and other enzyme systems. None of these changes significantly influences the proximate composition of the resultant meat.

The composition of beef, pork, lamb, veal, chevon, and horse meat, as shown in Table 17-1, reflects very few species differences that could influence microbial growth. Adequate protein, moisture, and other nutrients are available in the meat of each of the species to support microbial growth. The limiting factors then are the availability of the nutrients, the ultimate pH, the water activity, the availability of oxygen, the storage temperature, and the levels and types of microorganisms on the meat surface.

The pH of muscle at slaughter approaches neutrality but usually is lowered to about 5.5 due to the breakdown of glucose to lactic acid. When an animal is stressed prior to slaughter, the glucose and glycogen levels in the muscles are lowered antemortem, and less lactic acid is formed postmortem. If the pH of beef muscle remains above 6.2 post-slaughter, the carcass is considered a dark cutter. The resultant meat will be dark, firm, and dry (DFD). This DFD meat supports microbial growth better than meat from unstressed animals[53] and will spoil more rapidly.[36] These authors suggested that because of the very low concentrations of glucose and glucose-6-phosphate in DFD meat, the spoilage bacteria utilize amino acids as substrates, and unpleasant by-products result. Egan and Roberts[36] indicated that the growth rates of pseudomonads, the predominant spoilage bacteria at refrigeration temperatures, are relatively constant within the pH range of 5.5 to 7.0.

17.3 SLAUGHTER/PROCESSING

17.3.1 Inspection

Current "Good Manufacturing Practices in Manufacturing, Packing, or Holding Human Food" describes the sanitary and processing requirements necessary to ensure the production of wholesome food.[15] The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) evaluated 1,000 of the 6,400 meat and poultry establishments that they inspect and documented conditions that have previously been shown to result in high levels of contamination of meat with microorganisms. They have revised their "Sanitation Standard Operational Procedures" which outline standards of performance to obtain a wholesome product.[14] In addition, the UDSA has required the implementation of "The Hazard Analysis and Critical Control Point System" (HACCP) in inspected meat plants to improve the microbiological, chemical, and physical safety of meats.[127] The system is developed by plant management using guidelines established by the USDA. Under this preventative system, each plant determines steps in the process or the manufacture of the product where food safety problems might occur; then establish critical control points (CCP), critical limits, and corrective measures. They take corrective action when necessary, document and verify their action, and provide FSIS access to their records. Periodic testing for *Escherichia coli* and *Salmonella* spp. are part of the system. Additional information on HACCP is provided in Chapter 58.

Once slaughter animals have been transported to the abattoir, they are subjected to a pre-slaughter inspection.[14,121] Any dead, dying, diseased, disabled, or drugged (5D) animals are segregated. Carcasses from dead animals are denatured and not allowed for human consumption. Dying, diseased, disabled, or drugged animals undergo further evaluation and potential exclusion as meat for human consumption. Inspectors attempt to exclude meat of diseased animals from marketing channels and to minimize microbial contamination of meat. During antemortem and postmortem inspection, using subjective procedures such as visual appraisal and palpation, about 164,000 cattle (0.50%), 212,000 pigs (0.24%), and 13,000 sheep (0.28%) annually are excluded from marketing channels in the US, because of disease, unfitness, or adulteration.[119] Currently, research is under way to develop additional methods to detect specific disease conditions in the carcasses of slaughter animals and the resultant meat.

Unfortunately, slaughter animals are liable to be contaminated with fecal material. Such fecal material has been shown to contain *Salmonella* spp., *C. jejuni/coli*, and other pathogenic bacteria. These bacteria are not only in the intestinal tract, but also on the exterior of the slaughter animal. In 1994, the FSIS declared *Escherichia coli* O157:H7 an adulterant in ground beef, thus requiring that it must be excluded from such meat. In 1999, the FSIS proposed to extend the declaration of the bacterium as an adulterant if found in "non-intact" beef products such as mechanically tenderized, cubed, reconstructed, or injected products. In 1997, one company voluntarily recalled 25 million pounds of ground beef because illness caused by *E. coli* O157:H7 was traced back to their product. The resultant loss of confidence and financial drain caused the plant to be closed. FSIS is faced with the enormous task of excluding from market channels those carcasses that have not been cleansed adequately to remove the potentially pathogenic bacteria. Most slaughter animals have some degree of surface fecal contamination and therefore are exposed to pathogenic bacteria. However, dressing procedures significantly decrease microbial counts on the carcass.

17.3.2 Residues

Antibiotic and chemical residues, though of concern to the consumer, are rated below microorganisms in importance by food scientists. Rapid screening tests are in use in slaughter establishments for detecting some residues. Antibiotic residue detection methodologies based on the inhibition of bacterial growth require close scrutiny because some pork carcasses contain naturally occurring bacterial inhibitors that result in false positives in such screening tests.[87]

17.3.3 Control

The US FSIS has published guidelines for the application of HACCP to the processing of muscle into meat.[127] Figure 17-1 provides one example of a HACCP flow chart for the slaughter, fabrication, and packaging of cattle, whereas Figure 17-2 provides similar information for pigs. Each of these slaughter animal groups is subject to surface contamination with dung as a result of farm practices and stress during live transport, holding, and slaughter. Attempts are under way to limit the stress in livestock during shipping and holding prior to slaughter because stressed animals are more apt to shed pathogenic bacteria such as *Salmonella* spp. and can lead to contamination of greater numbers of animals. Efforts have been under way for a number of years to present animals for slaughter in a humane manner that minimizes animal stress and injury.[50–52]

Production practices are under review to try to minimize fecal contamination of livestock. Greater attention is now being directed toward eliminating fecal material from the surface of animals presented for slaughter. Pre-slaughter washes, and post-slaughter washes and hair removal, are being evaluated as a means of reducing the potential for contamination of the carcass during hide removal. However, carcasses from beef that had considerable quantities of dung on their hides have been shown to maintain higher levels of microbial contaminants than cleaner animals, even if the dirty animals were processed using a slower line speed and with greater care in slaughtering procedures.[99]

Feed withdrawal, before the transport of live animals to slaughter facilities, has the advantage of lowering gastrointestinal tract contents, reducing the incidence of viscera rupture and, therefore, lowering the risk of pathogen contamination of carcasses.[86]

Throughout the slaughter establishment, walls should be placed between operations to separate areas with high airborne microbial populations from those with fewer numbers. Kotula and Emswiler-Rose[71] found 2 to 3 Log_{10} aerobic cfu per 0.028 m^3 in the various processing rooms of a commercial pork processing establishment. In commercial beef processing establishments, Worfel et al.[132] reported 1.66 to 3.17 Log_{10} aerobic cfu per 0.028 m^3 in hide-on areas, with the lowest counts seen in a plant with a serpentine slaughter line. In hide-off areas counts ranged from 0.52 to 2.08 Log_{10} aerobic cfu per 0.028 m^3. Air filters should be utilized to reduce these counts, especially in locations where trimmed meat is being fabricated. Aerosols should be controlled throughout processing. Simple operations such as carcass spraying, carcass splitting, and clean up significantly increase the number of airborne microorganisms.

Slaughtered cattle, sheep, goats, and horses are skinned whereas most pig carcasses are scalded, dehaired, singed, and polished before evisceration. Potentially pathogenic bacteria can be found in fecal material on the surface of the slaughter animal and in its intestinal tract. Particular care is required for cattle, sheep, goats, and horses during skinning, head and shank removal, and bagging or tying of the bung to prevent fecal contamination of the carcass. Zink[133] reported that the use of steel plugs chilled in liquid nitrogen and inserted into the bung was an effective alternative to tying.

After slaughter, hogs are usually scalded in water between 60° and 70°C for 5 to 7 minutes for hair removal, rather than skinned (Figure 17-2). Bacteria that are potentially pathogenic to humans have been shown to accumulate in scald water and its use has been criticized.[59,112] Scalding allows microbially contaminated water to enter the carcass via the nose and mouth, and sticking point, and to be transferred to the lungs, circulatory system, organs, and musculature.[59,112] Scalding pigs hanging in the vertical position resulted in less internal contamination than combined scalding/dehairing. The use of steam scalding has been suggested.[133] Skinning of pork carcasses is more economical than scalding,[60] but the industry continues to have a market for hams with skin.

After pork carcasses are scalded, they are singed to remove any remaining fine hairs. Singeing has been reported to effectively reduce the bacterial numbers on the skin surface in some cases,[24,47,89] but increase it in others.[47,89]

The carcasses are then polished with brushes to remove charred portions of the epidermis (blackscraping); this process may spread bacteria over the carcass.[89,96] Ice blasting, ie, impelling small ice crystals against the singed carcass surface, was reported to remove the carbonized layer of the rind effectively and to cost about the same as using automated polishers.[133]

Pork carcasses that were brushed before scalding had higher counts of aerobic bacteria than nonbrushed carcasses; however, this increase was not apparent until after singeing and blackscraping.[96] The researchers' explanation is that the brushes break and lacerate the carcass skin, thereby allowing microorganisms to enter the skin during scalding and be protected from the heat of singeing. Blackscraping contributes further to contamination. Although there was no significant difference after chilling between brushed and nonbrushed carcasses, the authors strongly discouraged the use of prescalding brushing.

Though not currently utilized, postexsanguination chemical (sodium sulfide) dehairing of beef carcasses has been reported to reduce visual contamination; however, it did not reduce the overall bacterial load.[103] The potential commercial dehairing procedure calls for dehairing after stunning, but before bleeding. Delay of bleeding after stunning may be considered inhumane. Dehairing after bleeding may contribute to chemical contamination of edible portions of the carcass.

The carcass is trimmed to remove visible dirt, lesions, and visible bruises. If the viscera are ruptured during evisceration, further trimming and washing is needed. The incidence of ruptured viscera from hogs was shown to be reduced from 1.5% to 0.5% by increasing the rest period of the live animal after transport to 4 hours instead of 2 hours.[86] Cross-contamination between carcasses can be reduced if operators wash their hands between carcasses and if knives are decontaminated.[18] Efforts are under way to more completely automate evisceration with the expressed goal of minimizing cross-contamination during that process.[133]

17.3.4 Carcass Washing

The regulatory agencies, the meat industry, and the scientific community have directed much attention to cleansing procedures for reducing the number of bacteria on each meat carcass, regardless of species. Suggested procedures include treating carcasses with cold water, hot water, steam, chlorinated water (20 to 50 ppm), a 1% to 3% acetic, lactic, or citric acid solution, tripolyphosphate, potassium sorbate, ozone, irradiation, and others. A number of these procedures are not approved, and others are not feasible because of economic, palatability, or public health considerations.

Pressurized cold water, or water heated to 82°C, is effective in removing surface bacteria from carcasses. It effectively removes dirt that may have fallen onto the carcass during skinning. Water at 82°C can be utilized as a bactericidal treatment after evisceration. A series of warm water (57°C) and hot water (95°C) washes under pressure reduced the initial counts of 5 Log_{10} by 3.7 logs, 3.8 logs, and 3.3 logs, for *E. coli* O157:H7, *S. typhimurium*, and hydrotolerant coliforms, respectively.[13] The authors suggested that the use of coliform counts may be possible for verification of the effectiveness of hot water treatments used as critical control points (CCPs) in HACCP.

Steam treatment of beef carcasses has been reported to be more effective than trimming, 35°C water washing, or hot water/steam vacuum spot cleaning, in reducing counts of *E. coli* O157:H7, *S. typhimurium*, and *Listeria monocytogenes* on inoculated carcasses.[95] Steam treatment followed by spot cleaning can give a reduction of 4.2 to 5.3 Log_{10} cfu cm^{-2}.[95] Steam pasteurization of naturally contaminated carcasses, within a commercial slaughter facility that used knife trimming, steam vacuum spot cleaning, and final carcass washing, reduced populations of aerobic bacteria, *E. coli*, coliforms, and Enterobacteriaceae more than knife trimming, steam vacuum spot cleaning, and final carcass washing alone.[92] Whereas 15 of 79 cow carcasses and 11 of 81 cattle carcasses initially had surface counts of 0.6 to 1.5 Log_{10} cfu cm^{-2} of *E. coli*, none of the carcasses had viable *E. coli* (detection limit of 0.6 cfu cm^{-2}) after 6 or 8 seconds of steam pasteurization treatment.[92] One major beef company in the US has installed steam treatment units in each of their establishments. Steam, hot water spray washes, and a steam vacuum treatment of beef and sheep carcasses will decrease bacterial populations,[25,26,28] and could reduce the amount of trimming necessary.[25] Gill et al.[48] reported the bacterial numbers from randomly selected polished pork carcasses washed with 85°C water for 20 seconds to be 1.1 Log_{10} cm^{-2} for the loin-lumbar region, 0 for the loin-thoracic region, 1.0 Log_{10} cm^{-2} for the foreleg, and 0 for the belly, whereas the counts for the unwashed carcasses ranged from 2.4 to 3.3 Log_{10} cm^{-2}.

Beef carcasses and the resultant meat are subjected to the possibility of cross-contamination from the time the hide is removed until the retail cut has been packaged. Cutter et al.[20] hypothesized that if a carcass is treated with dry heat, such as 400°C for 15 seconds (administered with a propane forced-air heater), the surface connective tissue will shrink, become dehydrated, and may affect attachment of surface bacteria. Carcasses treated in this manner immediately after skinning might be less subject to surface contamination during other handling procedures. To test this concept, beef desiccated in that manner was then inoculated with feces containing aerobic spoilage bacteria (6.5 Log_{10}), *L. innocua* (4.0 Log_{10}), *C. sporogenes* (3.6 Log_{10}), *S. typhimurium* (2.6 Log_{10}), and *E. coli* O157:H7 (3.3 Log_{10}) to simulate gross contamination. The beef was then washed with water (125 psi, 35°C, 15 seconds) and desiccated (400°C, 30 seconds). This treatment reduced the aerobic count by about 3 Log_{10} and the bacteria mentioned above were reduced to undetectable levels of less than 1 cfu cm^{-2}. In the same study, a water wash (125 psi, 35°C, 15 seconds) alone on unheated carcasses reduced the numbers of each of the test bacteria by at least 2 Log_{10}. The authors conjectured that the final heat treatment raised the temperature of the water adhering to the beef surface to >65°C, thereby destroying the bacteria. Use of 300°C instead of 400°C minimized discoloration of the surface of the carcass and reduced desiccation without decreasing the effectiveness of the process.

Chlorine has been used in the dairy, flour, water, and other industries to destroy bacteria. Used properly, it is also effective in reducing the numbers of viable surface bacteria on carcasses. The bactericidal effectiveness of chlorine sanitizers is dependent upon the type and concentration of the chlorine compound used, pH, temperature, contact period, type of microorganisms, and presence of organic material.[5]

Organic material, such as lean muscle, adipose tissue, and serum albumin, will deplete aqueous chlorine levels.[77] Therefore, chlorine solutions need to be carefully monitored to avoid depletion, while staying as close to the legal maximum, to ensure effectiveness. In the US, the maximum chlorine level is usually 20 to 50 ppm.[125]

Of the four sources of aqueous chlorine, which include (1) gaseous chlorine bubbled through water, (2) chlorine dioxide, (3) calcium hypochlorite dissolved in water, and (4) chlorine generated electrolytically from sodium chloride, the electrolytically generated chlorine solutions appear to be most practical and effective, but the use of sodium or calcium hypochlorite may be more economical. Gaseous chlorine may be incorporated into water to a level of about 25 ppm but, depending on the water temperature, the chlorine may not be bound and may escape into the room. Such gaseous chlorine is not only irritating but is also deadly.

In a study where beef forequarters were washed with water, sodium hypochlorite, calcium hypochlorite, or chlorine dioxide, calcium hypochlorite was shown to be a little less effective than the sodium hypochlorite solutions generated electrolytically from brine, but the difference was not very great.[38] Chlorine dioxide was most effective against psychrotrophic bacteria at about 50 ppm (1 Log_{10} reduction in count after 24 hours), whereas plain water and calcium hypochlorite at 50 ppm caused no reduction in numbers of viable psychrotrophic bacteria (Table 17-2).[38] Reductions in aerobic counts were

Table 17-2 Mean Log_{10} Values[a] of Psychrotrophic Bacteria on the Surface of Market Beef Forequarters Before and After Chlorinated Wash

		Mean Log_{10} Counts cm^{-2}		
Chlorine Source	*Concentration (ppm)*	*Before Wash*	*1 hr After*	*24 hr After*
Biocidal electrolytic sanitizer (sodium hypochlorite)	0	3.2^{a-d}	3.7^{a-c}	3.3^{a-d}
	50	3.6^{a-d}	3.6^{a-d}	3.6^{a-d}
	100	3.2^{a-d}	3.4^{a-d}	2.2^{d-f}
	200	2.8^{a-d}	3.6^{a-d}	1.2^{f}
	400	3.2^{a-d}	3.4^{a-d}	1.4^{ef}
Calcium hypochlorite	0	3.0^{a-d}	3.6^{a-d}	3.0^{a-d}
	50	3.9^{ab}	4.0^{ab}	3.9^{ab}
	100	4.1^{ab}	3.2^{a-d}	3.4^{a-d}
	200	3.6^{a-d}	2.6^{b-f}	2.6^{b-f}
	400	4.1^{a}	3.6^{a-d}	3.3^{a-d}
Oxine (chlorine dioxide)	0	2.3^{c-f}	2.7^{a-e}	2.6^{b-f}
	50	3.6^{a-d}	3.7^{a-c}	2.7^{a-e}
	100	3.6^{a-d}	3.8^{ab}	3.4^{a-d}
	200	3.6^{a-d}	3.9^{ab}	4.0^{ab}
	400	3.4^{a-d}	3.4^{a-d}	2.6^{b-f}

[a] Each value is based on six forequarters per test cell. Data within columns or rows followed by the same letter or letters are not significantly different.

Source: Reprinted with permission from B.S. Emswiler, A.W. Kotula, and D.K. Rough, Bactericidal Effectiveness of Three Chlorine Sources Used in Beef Carcass Washing, *Journal of Animal Science*, Vol. 42, pp. 1445–1450, © 1976, American Society of Animal Science.

greatest when the chlorine solution was adjusted to a pH of 6 to 7 with acetic acid and the chlorine concentration was increased to 200 ppm (Table 17-3).[63] Lowering the pH to 4 or 5 decreased the effectiveness of the wash. A high-pressure wash of 24.6 kg cm^{-2}, which used 4 L to wash a beef forequarter for 30 seconds, was more effective than a wash at 4.2 kg cm^{-2}, which used 1 L. Wash water at 51.7°C yielded an average Log_{10} reduction of 3.2 compared with 2.3 for wash water at 12.8°C. The effects of pH and wash water temperature were overshadowed by the effectiveness of high-pressure washing, which frequently resulted in a reduction of the bacterial numbers to less than two per square centimeter (Table 17-3).[63]

Research by Emswiler-Rose and Kotula[41] on the inhibition of pure cultures of bacteria with chlorine indicates that use of 20 to 50 ppm chlorine, which represents the level usually allowed by the US FSIS, may have less than the desired effect on some bacteria, including *Salmonella* spp., *B. cereus*, *E. coli*, and *Y. enterocolitica* (Table 17-4). Admittedly, in that study, there was binding of some of the chlorine to the agar, making it unavailable to inhibit microorganisms. However, chlorine also binds with organic material, such as lean and adipose tissue, during usage in slaughter and processing facilities. Available chlorine is reduced most readily by lean muscle, then by agar, and least by adipose tissue,[77] therefore, the agar plates used by Emswiler-Rose and Kotula[41] offered a reasonable approximation of how the chlorine will act in a slaughter/processing situation. Chlorine solutions are used, and approved by FSIS, based on initial concentration, that is, before contact with meat.

Although chlorine has been used to control microbial growth on animal tissues for over 40 years in the US, questions persist concerning the safety of chlorine treatments of meat. Fidler[43] reported that hyperchlorinated water (25 to 30 ppm) suppresses macrophages, may compromise host resistance against neoplasms, and may result in anomalies in animals. Chlorine in the presence of humic substances can form carcinogenic compounds. Some fatty acids and possibly some amino acids of meats, react with aqueous chlorine.[62] Chlorinated oleic acid decreased absorption in the digestive tracts of rats from 93% to 76%.[19]

The use of organic acids has been studied extensively and has been recommended by numerous scientists for the reduction in colony-forming units on carcasses.[2,3,108,131] However, organic acids, such as acetic and lactic, caused a bleaching of the meat surface in experimentally treated retail cuts without adequately reducing bacterial counts.[76] In many published reports the reduction is about 90%. From an economic standpoint, one may question the logic of using organic acids if the resultant reduction is only about one logarithm when the total count is about 5

Table 17-3 Total Aerobic Microorganisms[a] from Market Beef Forequarters After Chlorinated Wash

			Sampling Time		
Pressure ($kg\ cm^{-2}$)	Temperature (°C)	pH	Before (cm^{-2})	45 min After (cm^{-2})	24 hr After (cm^{-2})
4.2	12.8	4	450	120	69
		5	140	67	130
		6	1200	630	14
		7	1900	26	4
	51.7	4	380	270	9
		5	2200	39	10
		6	6600	91	36
		7	2400	140	140
24.6	12.8	4	620	12	<2
		5	1200	9	9
		6	4400	100	<2
		7	3300	16	<2
	51.7	4	1700	18	<2
		5	5000	85	23
		6	8600	3	<2
		7	6500	27	<2

[a] Each value is based on six forequarters washed with 200 ppm chlorine after evisceration. Washes with 4.2 kg cm^{-2} pressure used 1 L of solution, washes with 24.6 kg cm^{-2} pressure used 4 L of solution.

Source: Reprinted with permission of A.W. Kotula et al., Beef Carcass Washing To Reduce Bacterial Contamination, *Journal of Animal Science*, Vol. 39, pp. 674–679, © 1974, American Society of Animal Science.

or 6 logarithms. In other studies, lactic or acetic acid (1.5% or 3%) or 12% trisodium phosphate, which initially reduced pseudomonads by less than 1 Log_{10}, then provided a residual inhibitory effect so that whereas bacteria on the control samples and water-washed samples increased to 5 Log_{10} in 19 days of vacuum packaging, the samples treated with the antimicrobials did not increase in number.[27] Of equal interest, the antimicrobials reduced numbers of *E. coli* O157:H7, *L. innocua*, and *C. sporogenes* by about 3 Log_{10} and inhibited the growth of these bacteria during 19 days of vacuum packaged storage.[28] There appeared to be little difference between using 1.5% and 3% of the antimicrobials, but application method appears important. Previous literature[30] reported that *E. coli* O157:H7 on beef carcasses was exceptionally tolerant to this treatment. Citric, acetic, or lactic acid at concentrations of 0.5% to 1.5% applied at 22° and 55°C resulted in a reduction of less than 0.5 Log_{10} of *E. coli* O157:H7. Trisodium phosphate was not as effective in reducing numbers of *L. innocua* as were acetic and lactic acid. A soapy mouth feel has been attributed to meat treated with trisodium phosphate. Repeated pressured water washes might be more efficient and would not add to consumers' apprehension about the use of chemical additives but the residual inhibition of growth would not be evident.

The use of potassium sorbate has been studied for the control of bacteria on fresh red meat.[113] When studied for the control of bacteria on bacon, it caused prickly mouth sensations and "chemical"-like flavors,[6] swelling of the hands of individuals handling the treated meat, and asthma-like symptoms during cooking and after consumption.[55] There is no reason to expect dissimilar results if potassium sorbate were used on red meats. Additionally, potassium sorbate, in combination with nitrite (from vegetables, cured products, or saliva) and heat can form mutagenic compounds.[55]

17.3.5 Irradiation

Irradiation of pork, using a minimum dosage of 0.3 kGy and a maximum dosage of 1.0 kGy, has been authorized in the US for the control of *Trichinella spiralis*.[93] A minimum of 1.5 kGy and a maximum of 3.0 kGy are allowed for the control of pathogenic bacteria on poultry. The use of irradiation for the destruction of bacteria on red meats has been approved by the Food and Drug Administration but dosage levels have not yet been specified by the USDA. It is recommended that any irradiation treatment should be such that it will allow some competitive bacteria to survive and help control the germination of spores of *C. botu-*

Table 17-4 Lowest Concentration (ppm) of Aqueous Chlorine at Which Inhibition Occurs, As Determined by Regressing Equations to Zero Zone of Inhibition

	Chlorine Source (ppm)	
Bacteria	*Hypochlorous Acid*	*Chlorine Dioxide*
Achromobacter pestifer	219	1065
Bacillus cereus	242	217
Campylobacter jejuni	78	460
Clostridium perfringens	53	8
Escherichia coli	349	629
Micrococcus varians	195	170
Proteus bulgaris	123	582
Pseudomonas aeruginosa	307	1242
Pseudomonas cepacia	168	867
Pseudomonas fluorescens	76	128
Pseudomonas fragi	84	225
Salmonella enteritidis	285	896
Salmonella newport	290	1359
Salmonella saint-paul	306	1346
Salmonella typhimurium	362	758
Staphylococcus aureus	241	339
Streptococcus faecalis	389	286
Yersinia enterocolitica	177	564
Mixed culture from beef	280	1187
Mixed culture from chicken	319	1419

Source: Reprinted with permission from B.S. Emswiler-Rose and A.W. Kotula, Inhibition of Bacterial Growth by Two Chlorine Sources in a Model System, *Journal of Food Science*, Vol. 49, pp. 931–933, © 1984, Institute of Food Technologists.

linum, which could result in toxin production.[93] The same concerns about the potential for spore germination have prevented the use of anoxic environments, including modified atmosphere packaging (MAP) or vacuum packaging for irradiated poultry. Some countries other than the US have approved irradiation of red meats at maximum doses of 3–7 kGy.

It has been estimated that irradiation of a pork carcass would cost 3 to 8 dollars, in addition to the cost of transporting the carcass to and from the irradiation facility. If the pork carcass is to be irradiated with gamma rays, it is desirable to freeze the pork first to minimize the off-odors and off-flavors that result from the irradiation at dosages of 1 kGy and higher. Under suitable conditions, freezing of pork destroys viable *T. spiralis*. The temperature at which pork needs to be frozen to minimize off-odors and off-flavors during irradiation (–60°C) is lower than that required to inactivate *T. spiralis* (–37.2°C for 0.5 hour). In the US, a pork certification program exists, which ensures that the raw pork has received a proper freezing treatment (Tables 17-5 and 17-6).

The irradiation dose that is effective against spoilage bacteria must be increased to control potentially pathogenic bacteria of importance, and higher doses are required for frozen red meats than for chilled meat.[93] Unfortunately, increased dosages cause an increase in development of off-flavors and odors. Although irradiation is authorized for the control of *T. spiralis* in pork and for the control of pathogenic bacteria in poultry, its use is very limited in the US. Regulations require that any such irradiated meat and poultry be labeled as having received that treatment.

In the US consumers have not yet accepted irradiation of meat and poultry. Those consumers who show interest in irradiated meats seem to have the incorrect perception that these products would be sterile, and therefore, the consumer would not need to be concerned with time/temperature abuse of these products. This is an extremely dangerous attitude because meats irradiated at the currently accepted level are not sterile, and could be potentially hazardous if handled improperly.

17.3.6 Fabrication

After a beef, pork, or lamb carcass has been eviscerated and washed, it is customarily chilled to below 4°C within 24 hours. This reduces the rate of growth of surface bacteria, slows enzymatic activity,

Table 17-5 Required Period of Freezing of Pork at Temperatures Indicated To Destroy *Trichinella spiralis*, for Certified Pork in the US

Temperature °F	*Temperature °C*	*Group 1[a] (days)*	*Group 2[b] (days)*
5	–15.0	20	30
–10	–23.3	10	20
–20	–28.9	6	12

[a] Group 1 comprises product in pieces, layers, or within containers not exceeding 15.2 cm (6 inches) in thickness or depth of product.

[b] Group 2 comprises product in pieces, layers, or within containers that exceeds 15.2 cm (6 inches) in thickness, but does not exceed 68.6 cm (27 inches).

Source: Reprinted from *Code of Federal Regulations*, Title 9: Animals and Animal Products, Chapter III: Food Safety and Inspection Service, Meat and Poultry Inspection, 1995, U.S. Department of Agriculture.

Table 17-6 Alternate Periods of Freezing of Pork at Temperatures Indicated To Destroy *Trichinella spiralis*, for Certified Pork in the US

Maximum Internal Temperature		
Degrees Fahrenheit	*Degrees Centigrade*	*Minimum Time*
0	−17.8	106 hours
−5	−20.6	82 hours
−10	−23.3	63 hours
−15	−26.1	48 hours
−20	−28.9	35 hours
−25	−31.7	22 hours
−30	−34.5	8 hours
−35	−37.2	0.5 hours

Source: Reprinted from Code of Federal Regulations, Title 9: Animals and Animal Products, Chapter III: Food Safety and Inspection Service, Meat and Poultry Inspection, 1995, U.S. Department of Agriculture.

and decreases biochemical reactions within the meat. A number of disadvantages are associated with the chilling of the intact carcass. Chilling occurs much more rapidly in smaller pieces, such as primals and trimmings, than in deep muscles of the intact carcass. Removal of excess fat and bone before chilling could save considerable energy in terms of total refrigeration. In addition, "breaking" (cutting into primal cuts) a chilled carcass requires more energy than breaking a hot carcass because chilled meat is less flexible. No differences of any practical significance were found between aerobic plate counts, coliforms, or *E. coli* in ground beef fabricated from trimmings that were hot-boned (excised from the carcass within 2 hours postmortem) and trimmings that were cold-boned (removed after 24 hours chill) at 3°C.[40,65] It was necessary to add CO_2 snow to the hot-boned trimmings during the fabrication of the ground beef to reduce the meat temperature rapidly and preclude smearing of the fat during grinding. Hot-boned meat can be distributed to the consumer faster.

In the US, graded beef usually originates from breeds such as Hereford or Aberdeen Angus cattle specifically raised for their meat. Hot-boning of beef carcasses would occur after eviscerating and washing but before chilling. There are disadvantages to hot-boning because it can result in toughening of the meat and changes in the appearance of the meat create difficulties in grading. This problem can readily be resolved by electrical stimulation of the carcass immediately after evisceration and washing. When properly stimulated, the carcass shows a rapid drop in pH (Figure 17-3), which prevents shortening and toughening of the meat.

One of the greatest obstacles that must be overcome during the breaking of carcasses is the cross-contamination between carcasses and from equipment that occurs when quarters, primals, and subprimals are placed on trays or conveyor belts.[65] From a bacteriological standpoint, the primal should be placed into a package as soon as it is excised from the carcass.

More than 50% of the beef sold in the US is sold as ground beef. The grinding and mixing involved in its manufacture distribute bacteria normally found on the meat surfaces throughout the entire product. The bacteriological quality and shelf life of ground beef depend upon the bacteriological quality of the ingredients, sanitation, and temperature during processing, type of packaging, and time and temperature of storage. This was demonstrated by a study in a centralized meat packaging and distribution center that prepared about 18,000 kg of ground beef daily under federal inspection.[17] Table 17-7 shows the level of selected bacteria on the ingredients of the ground beef and the final product. Surface bacterial counts were higher on the cow beef than on trimmings or flank meat. The meat juices in the bottom of the stainless steel holding bins also contained inordinately high bacterial counts. As the bins were filled, the juices were squeezed from the meat, washed over the surface of other pieces, and provided a good substrate for growth. The bacteriological quality of cow beef and expressed juices was significantly correlated with the bacteriological quality of the resultant ground beef (Table 17-7).[17] The addi-

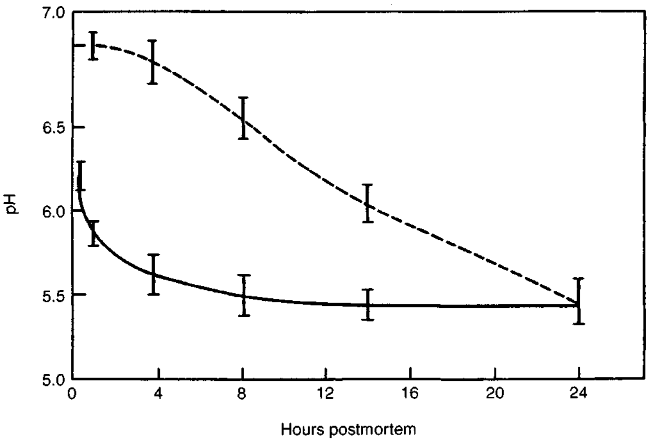

Figure 17-3 pH Change in Electrically Stimulated (solid line) and Nonstimulated Beef (dotted line). *Source:* Reprinted with permission from A.W. Kotula and B.W. Berry, Advances in Refrigeration of Meat and Meat Products, *International Journal of Refrigeration,* Vol. 4, pp. 340–344, © 1981, Elsevier Applied Science.

Table 17-7 Mean Log$_{10}$ Counts[a] of Bacteria in Ground Beef Ingredients and Final Product

Product	*Sample Unit*	*Total Aerobes*	*Psychrotrophs*	*Coliforms*	E. coli	S. aureus	C. perfringens
Line trimmings (top layer)	cm^2	3.93	4.03	0.31	0.19	−0.39	−0.82
Line trimmings (bottom layer)	cm^2	4.03	4.01	−0.01	−0.19	−0.75	−1.00
Cow beef (top layer)	cm^2	4.23	3.12	0.43	−0.05	−0.31	−0.93
Cow beef (bottom layer)	cm^2	5.20	5.66	1.52	−0.05	0.17	−0.44
Flank meat	cm^2	3.06	2.78	−0.53	−0.48	−0.75	−1.00
Juices from line trimmings	ml	5.49	5.77	2.05	0.34	−0.75	−0.59
Juices from cow meat	ml	5.64	6.60	1.63	0.88	0.63	−0.37
Flaked meat #1	g	3.92	2.95	0.85	0.49	0.24	−0.75
Flaked meat #2	g	3.88	3.10	0.73	0.35	−0.13	−0.75
Coarse grind of line trimmings	g	3.99	3.80	0.47	0.11	0.23	−0.87
Coarse grind of cow meat	g	3.63	4.15	0.85	0.24	−0.38	−0.63
Fine grind #1	g	4.57	4.84	1.72	1.22	0.73	−0.51
Fine grind #2	g	4.63	4.87	1.59	0.98	0.36	−0.50

[a] Each value is the mean of eight samples (4 days × 2 batches/day).

Source: Reprinted with permission from C.M. Chestnut et al., Bacteriological Quality of Ingredients Used in Ground Beef Manufacture, *Journal of Animal Science*, Vol. 44, pp. 213–217, © 1977, American Society of Animal Science.

tion of chilled plate cuts of beef, which had greater numbers of bacteria than the other ingredients, had an important influence on the bacteriological quality of the ground beef.[70]

17.4 INITIAL MICROFLORA OF FRESH RAW MEATS

17.4.1 Spoilage Bacteria

The important spoilage bacteria of fresh meat include *Pseudomonas*, *Acinetobacter/Moraxella*, *Aeromonas*, *Alteromonas putrefaciens*, *Lactobacillus*, and *Brochothrix thermosphacta*.[21,79] Emswiler et al.[39] cited literature that reported that ground beef may contain extensive bacterial populations. Although beef color change from red to brown can be indicative of microbial growth,[100] some bacteria can reverse this change. *Brochothrix thermosphacta*,[7] a fluorescent strain of *Pseudomonas* isolated from fresh meats,[42] and *Staphylococcus carnosus* and *Staphylococcus caseolyticus* isolated from fermented meats[88] can all cause reversion of the brown-colored metmyoglobin to the more desirable color of oxymyoglobin in meat products that showed sensory spoilage.

Lambert et al.[79] described the substrates used for growth and the metabolic by-products of major spoilage microorganisms (Table 17-8). Typically, spoilage bacteria use glucose, amino acids, and lactic acid, in that order, to support their growth. The availability of these nutrients to bacteria is restricted to some degree by connective tissue and adipose tissue, which envelope muscle, but the nutrients become more readily available when muscle is cut.

The major end-products of metabolism of common meat spoilage organisms are sulfides, amines, acetic acid, lactic acid, isovaleric acid, isobutyric acid, esters, and nitriles. During prolonged storage of beef products under vacuum, bacterial proteolysis and decarboxylation can release pressor amines, such as tyramine.[107] Such amines may be toxic to individuals taking monoamine-inhibiting drugs. A thorough review of the chemistry of spoilage of meat stored aerobically and that stored under vacuum was presented by Dainty et al.[22]

The growth characteristics of the spoilage bacteria of meat depend upon initial flora, level of contamination, storage time, and temperature.[4] In a classic study, Ayres[4] demonstrated that pronounced off-odor developed on the surface of meat when the microbial count reaches about 10^7 cm^{-2}, and slime developed at about 10^8 cm^{-2} (Figure 17-4). The onset of off-odors has been reported at 1 to 2 × 10^6 organisms cm^{-2}.[4,37,78] Meat with a greater initial level of contamination spoiled more rapidly and spoilage occurred more rapidly at higher temperatures (Figures 17-4 and 17-5). To extend the shelf life of meat, keep it clean, covered, and cold.

17.4.2 Potentially Pathogenic Bacteria

The important potentially pathogenic bacteria that have been linked to fresh red meat include *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Clostridium botulinum*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Clostridium perfringens*, *Campylobacter*, *Aeromonas hydro-*

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Table 17-8 Substrates Used for Growth and Metabolic By-Products of Major Meat Spoilage Microorganisms

	Substrates Used for Growth[a]		*Major End-Products of Metabolism*	
Microorganism	*Aerobic*	*Anaerobic*	*Aerobic*	*Anaerobic*
Pseudomonas	Glucose^{1} Amino acids^{2} Lactic acid^{3}	—	Slime Sulfides, esters, acid, amines	—
Acinetobacter/ Moraxella	Amino acids^{1} Lactic acid^{2}	—	Esters, nitriles, oximes, sulfides	—
Alteromonas putrefaciens	Glucose^{1} Amino acids^{1,2} Lactic acid^{3}	Glucose^{1} Amino acids^{1}	Volatile sulfides	H_2S
Brochothrix thermosphacta	Glucose^{1} Amino acids^{2} (glutamate only)	Glucose^{1}	Acetic acid Acetoin Isovaleric acid Isobutyric acid	Lactic acid, volatile fatty acids
Enterobacter	Glucose^{1} Glucose 6-phosphate^{2} Amino acids^{3} Lactic acid^{4}	Glucose^{1} Glucose 6-phosphate^{2} Amino acids^{3}	Sulfides Amines	Lactic acid CO_2, H_2 H_2S Amines
Lactobacillus	—	Glucose^{1} Amino acids^{2}	—	Lactic acid, volatile fatty acids

[a] The number in superscript indicates the order of utilization of this substrate.

Source: Reprinted with permission from A.D. Lambert, J.P. Smith, and K.L. Dobbs, Shelf Life Extension and Microbiological Safety of Fresh Meat—A Review, *Food Microbiology*, Vol. 8, pp. 267–297, © 1991, Academic Press, Inc.

phila, and *Bacillus cereus*.[79,84,85] The USDA sampled approximately 2,000 steer/heifer, 2,100 cow/bull, and 2,100 pig carcasses, in a nationwide microbiological baseline data collection program (Tables 17-9 and 17-10). The incidence of the tested organisms on cow/bull carcasses was coliforms, 32%; *E. coli*, 16%; *S. aureus*, 8%; *C. perfringens*, 8%; *L. monocytogenes*, 11%; *C. jejuni/coli*, 1%; *Salmonella*, 3%; and *E. coli* O157:H7, 0%.[123] Rice et al.[98] reported an incidence of 3.4% for *E. coli* O157:H7 in fecal material of 205 cull dairy cows from 19 herds sampled on the farms, and 3.9% positive when 103 cull cows from 15 herds were sampled at slaughter. When 89 cull cows were sampled both on the farm and at slaughter, 3.3% were positive at the farm, 2.2% at slaughter, and 2.2% at both locations. The fact that one study isolated no *E. coli* O157:H7 on carcasses, whereas another study did find the organisms in the fecal material, emphasizes herd to herd variability and suggests that efforts are being taken during slaughter operations to minimize fecal cross-contamination. That cross-contamination of facilities and product does occur has been demonstrated (Table 17-11).[81] In a study of 30 dressed lamb carcasses in Spain, Sierra et al.[105] described isolating *Salmonella* (10%), motile aeromonads (33%), *Yersinia enterocolitica* (20%), *Campylobacter* (0%), and cold-tolerant *E. coli* (57%). None of the *Y. enterocolitica* were presumptive virulent and the O157 antigen was not detected in any of the *E. coli* isolates. Sampling of 50 pigs at a USDA-inspected slaughter establishment resulted in finding 2 with cecal contents, and feces were positive for serotype O:5,27, biotype 3 of *Yersinia enterocolitica*, whereas all other pigs were negative for pathogenic serotypes.[75] Other chapters in this book deal more specifically with pathogenicity, the growth characteristics, and public health importance of these potentially pathogenic bacteria.

Differences in plant operations as well as sampling procedures may influence bacterial results. Twenty samples of carcass, clod, and lean trim were taken in each of two plants slaughtering steer and heifers.[61] All samples were negative for *E. coli*

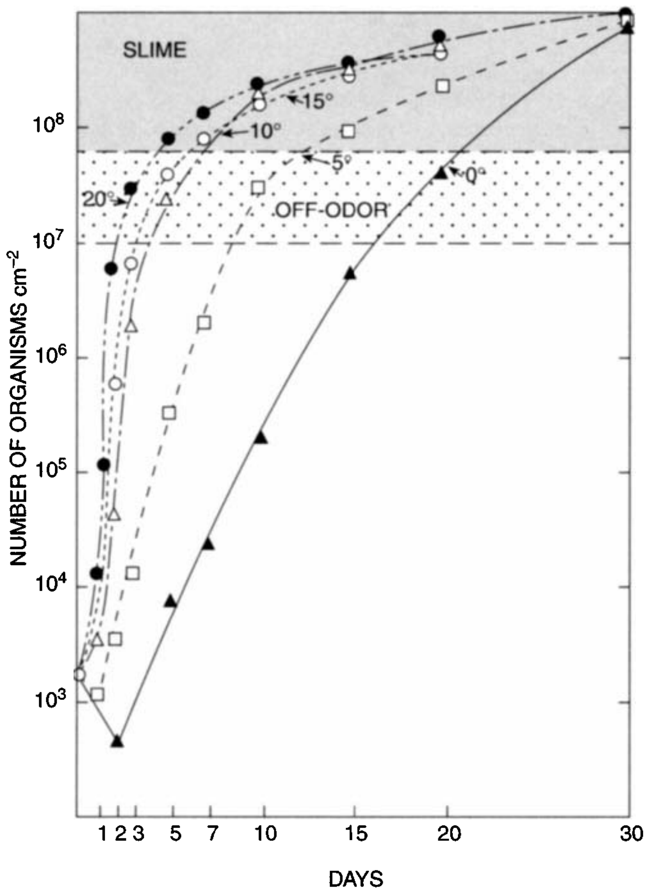

Figure 17-4 Rates of Growth of Organisms on Beef Stored at 0°, 5°, 10°, 15°, and 20°C. *Source:* Reprinted with permission from J.C. Ayres, Temperature Relationships and Some Other Characteristics of the Microbial Flora Developing on Refrigerated Beef, *Food Research*, Vol. 25, pp. 1–18, © 1960, Journal Paper No. J-3605 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1264.

O157:H7 and *Salmonella* spp. All samples were negative in one plant sampled for *C. jejuni/coli*. In the second plant, 27.5% of the carcass samples had detectable counts of *C. jejuni/coli* (greater than 10 cfu cm^{-2}), whereas the clod and trim had none. Five percent of the carcasses, 5% of the clod, and 8% of the trim samples had more that 10 cfu cm^{-2} or g^{-1} of *C. perfringens*. Twenty-one of 40 carcass samples and 15 of 40 clod samples had more than 100 cfu cm^2 or g^{-1} of coliforms. Coliforms and *E. coli* are indicators of fecal contamination, thus suggesting that improved sanitation might be helpful. The objective of the study[61] was to try to standardize sampling procedures to be used throughout the meat industry. Without standardization, results such as those presented by Karr and the USDA are of interest and provide a baseline but cannot be considered indicative of conditions in other establishments. Carcasses from cull cows, used for manufacturing ground beef, differed in incidence and counts of coliforms and *E. coli* among the six breaking plants which were sampled.[49] In plant A, 12 of 24 carcasses had detectable coliforms, and 7 of 24 carcasses had detectable *E. coli*, whereas in four of the other plants, 23 or more carcasses out of 24 sampled had detectable coliforms and *E. coli* present (detectable level of 1 cfu g^{-1}). The estimated values for the Log_{10} mean numbers of *E. coli* and coliforms was less than 1.0 for plant A but ranged from 1.2 to 3.0 for coliforms and 0.7 to 2.6 for *E. coli* in the other five plants sampled.[49]

When briskets and ground beef were examined from 547 slaughter establishments in the US, no association was found between *Salmonella* contamination and slaughter volume.[56] Brisket contamination with *Salmonella* was 0.9% of 1,836 heifers,

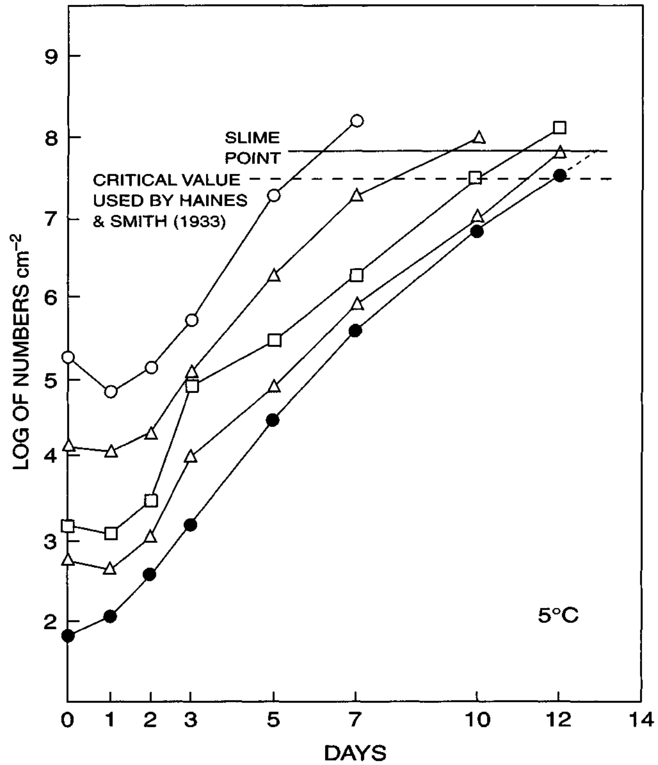

Figure 17-5 Relation of Initial Bacterial Load to Time Required for Development of Slime on Meat Stored at 5°C. *Source:* Reprinted with permission from J.C. Ayres, Temperature Relationships and Some Other Characteristics of the Microbial Flora Developing on Refrigerated Beef, *Food Research*, Vol. 25, pp. 1–18, © 1960, Journal Paper No. J-3605 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1264.

Table 17-9 Microbiological Baseline Data (percent incidence) of Red Meat Carcass Halves After 12 Hours Chilling and Ground Beef After Grinding and Before Packaging[118,123,124,126]

Microorganism	*Steers/Heifers*[a]	*Cows/Bulls*[b]	*Pork*[c]	*Ground Beef*[d]
Indicator bacteria:				
Aerobic plate count (@ 35°C)	99	100	100	100
Total coliform	16	32	45	92
Escherichia coli (biotype 1)	8	16	31	76
Pathogenic bacteria:				
Campylobacter jejuni/coli	4	1	31	0.002
Escherichia coli O157:H7	0.2	0	0	0
Salmonella spp.	1	3	9	8
Clostridium perfringens	3	8	10	53
Staphylococcus aureus	4	8	16	30
Listeria monocytogenes	4	11	7	12

[a] 2,000 samples.
[b] 2,100 samples.
[c] 2,100 samples.
[d] 563 samples.

1.1% of 1,239 bulls, and 5.0% of 397 calves, when the sample size was 25 g. When the sample size was 100 g the incidence was 1.2% of 1,607 heifers, 3.0% of bulls, and 7.9% of calves.[56]

Whereas it would be expected that the slaughter/processing procedures will result in a decrease in microbial contamination, an evaluation of cow carcasses in three plants showed an increase in fecal coliform counts in two of the three plants as the carcasses proceeded from dressing to trimming/washing, refrigeration, and cutting.[16] To obtain microbiological baseline data for ground beef, the USDA evaluated 563 samples. They did not find any *E. coli* O157:H7 (Table 17-9). Doyle and Schoeni[29] isolated *E. coli* O157:H7 from 3.7% of beef, 2.0% of lamb, and 1.5% of pork samples from retail stores in Wisconsin. Similar retail meats from Alberta, Canada, yielded isolates from 31% of the beef and 7% of the pork samples.

The incidence of *C. jejuni/coli* in meats at retail was as follows: pork chops, 5%; beef flank, 4.7%; ground beef, 3.6%; and stew lamb, 8.1% in a countrywide survey in the US involving 1,800 red meat samples.[111] A survey of 1,077 retail samples in the

Table 17-10 Microbiological Baseline Data of Red Meat Carcass Halves After 12 Hours Chilling (mean cfu cm^{-2}) and Ground Beef After Grinding and Before Packaging (mean cfu g^{-1})[118,123,124,126]

Microorganism	*Steers/Heifers*[a]	*Cows/Bulls*[b]	*Pork*[c]	*Ground Beef*[d]
Indicator bacteria:				
Aerobic plate count (@ 35°C)	475	1,130	4,900	7,900
Total coliform	35	40	77	96
Escherichia coli (biotype 1)	35	33	76	54
Pathogenic bacteria:				
Campylobacter jejuni/coli	0.1	0.1	0.1	ND[e]
Escherichia coli O157:H7	0.6	0	0	ND
Salmonella spp.	0.1	0.3	0.2	0.05
Clostridium perfringens	45	47	71	67
Staphylococcus aureus	24	25	84	31
Listeria monocytogenes	0.2	0.3	0.3	3

[a] 2,000 samples.
[b] 2,100 samples.
[c] 2,100 samples.
[d] 563 samples.
[e] ND = Not detected.

Table 17-11 Incidence (%) of Selected Bacteria in Live Hogs, Their Carcasses, and Slaughter Facilities

Sample	*Number*	Salmonella *spp.*	Campylobacter jejuni	Campylobacter coli	Yersinia enterocolitica
Feces	200	18	0.5	99	18
Diaphragms	200	1.5	2	20.5	2.5
Slaughter floor	16	25	NS[a]	NS	0
Scald water	16	0	NS	NS	0
Cold room	16	12.5	NS	NS	6.2
Total	448				

[a] NS = Not sampled.

Source: Adapted with permission from A.A. Mafu et al., The Incidence of *Salmonella, Campylobacter,* and *Yersinia Enterocolitica* in Swine Carcasses and the Slaughterhouse Environment, *Journal of Food Protection,* Vol. 52, pp. 642–645, © 1989, International Association of Milk, Food and Environmental Sanitarians, Inc.

United Kingdom (UK), evaluated for *C. jejuni/coli/laridis,* showed an incidence of beef, 23.6%; pork, 18.4%; lamb, 15.5%; liver, kidney, heart (all species), 47%.[44] Roberts et al.,[101] sampling ground beef from three supermarkets, three intermediate-sized stores, and three small family butchers in the UK during warm and cool weather found no significant or important store or seasonal effects. They reported that of their 162 samples, the mean Log_{10} count was 4.6 for Enterobacteriaceae, 2.46 for fecal streptococci, 2.6 for presumptive coliforms, 0.63 for *Clostridium perfringens,* and 0.5 for *S. aureus.* These studies to provide baseline data of red meats at retail demonstrated great variability in incidence but confirmed that pathogenic bacteria are present in the world's meat supply at the retail level.

Differences in reported incidence of pathogens are dependent on animal class (ie, cows, steers, heifers, bulls, calves), animal health, production practices, season, weather, transport and handling methods, slaughter and processing, microbiological methods (sampling, transport, holding, enrichment, media, incubation), statistical evaluation, and scientific interpretation of the data. Although *Salmonella* were detected in the USDA Microbiological Baseline Data Surveys,[118,123,124,126] the serotypes present may not have been pathogenic. Even with the 1% incidence of *C. jejuni/coli* in those surveys, one does not know the virulence of the strains, thus the actual risk to the consuming public cannot be adequately defined from these baseline incidence data.

In developing countries lacking adequate refrigeration facilities, the relative importance of the mesophilic microorganisms will be paramount compared with the psychrotrophic microorganisms that would predominate on refrigerated meat. This is not to say that one is more apt to become ill from the meat that has not been refrigerated, but additional care is indicated.

Typically, when refrigeration is lacking, animals are slaughtered in the early morning and the carcass is not cut up until a customer is prepared to buy some meat. Cuts are then sold sequentially from one end of the carcass to the other, thus, the exposed surface of the meat is kept to its smallest area so that contamination is minimized. Additionally, the meat is cooked soon after purchase so the bacteria do not have as much time to multiply.

17.4.3 Molds and Yeasts

The six genera of molds most readily isolated from slaughter animal carcasses or cuts include *Thamnidium, Mucor,* and *Rhizopus,* which produce white mycelia on beef; *Cladosporium,* which is associated with black spot; *Penicillium,* which produces green growth; and *Sporotrichum,* which produces white spot. The presence of mold is usually associated with dry meat that has an acid pH. In fresh meat, bacterial growth inhibits molds that might be present. The white mycelia of *Thamnidium* on aged beef has been considered desirable by some in the industry because beef aged 2 to 4 weeks at about 4°C has a particular "nutty" flavor, is very tender, and is preferred by some people.

The yeasts that have been isolated from meat include the genera *Candida, Torulopsis,* and *Rhodotorula.* Although sometimes present, these yeasts have not been a problem to the meat industry because when their requirements for growth are encountered during the storage of meat, bacterial spoilage occurs before the yeast numbers become a matter of concern.

17.4.4 Viruses

Fortunately, viruses that cause disease in slaughter animals are usually unable to cause disease in humans. Contamination by a food handler can result in transmission of viruses on meat. Enteric viruses that are transmitted by the fecal-oral route are discussed in Chapter 52. Viruses that cause hepatitis B, genital herpes, and AIDS are not believed to be transmissible by meat.

17.4.5 Parasites

Parasites of importance to the meat industry include nematodes like *Trichinella spiralis*, cestodes like *Taenia*, and protozoa like *Toxoplasma gondii*. Each of these parasites can be destroyed easily by proper cooking of the meat.

Trichinella spiralis

Although the incidence of *T. spiralis* in meat has been reduced significantly throughout the world, it is still liable to be present. In the US, one estimate suggests that one in a thousand hogs contain trichina.[58] Bears, rodents, wild pigs, hyenas, and other carnivorous animals also contain the parasite.

Many countries require mandatory inspection of pork carcasses for the presence of *T. spiralis*. In the US inspection by the pooled digestion technique or freezing the pork to destroy the parasite is required only if the meat is to be used in products that will not receive a thermal or curing treatment adequate to destroy the trichinae.

Current methodology for the detection of *T. spiralis* larvae in pork carcasses utilizes samples of the diaphragm muscle or tongue tissue. The diaphragm contains the second greatest number of larvae and is used most frequently because it is readily excisable and is of little economic importance. Sampling of the tongue, which has the greatest number of larvae, would potentially result in a defect that could reduce its value.

The USDA FSIS requires a minimum of 5 g of diaphragm muscle or tongue tissue, or a minimum of 10 g of other skeletal muscle tissue for the pooled digestion technique for detecting *T. spiralis* larvae in pork.[14] Under the assumption of the completely random series or Poisson distribution, 5 g samples (Figure 17-6) are expected to detect 99.3% of the carcasses with a larval density of 1 per g of muscle from the pillar of the diaphragm (Crus muscle). The Council Directive No. L 26/67 of the European Union (EU)[35] authorized the use of a number of sampling techniques including a pooled sample consisting of 100 samples of 1 g so that 100 carcasses can be evaluated simultaneously.

In both descriptions of sampling procedures (FSIS and EU), the various anatomical portions of the diaphragm muscle are assumed to contain a uniform distribution of *T. spiralis* in infected carcasses. However, Kotula et al.[72] evaluated five identifiable parts of the diaphragms from 30 infected pigs and reported the incidence of larvae to be the greatest in the Crus muscle and then in descending order, the Dorsal Pars costalis, the Pars sternalis, the Ventral Pars costalis, and the Lumbocostal arch. The use of a 1 g sample from each carcass, as recommended by the EU, may also be subject to question because it may detect only 63.2% of the carcasses with a larval density of 1 per g of muscle (Figure 17-6). Similarly, the trichinoscope, which utilizes 14 pea-sized samples of the diaphragm crushed between two pieces of glass and evaluated microscopically, may lack precision because of worker fatigue and other technical problems. More rapid and accurate procedures need to be developed for the detection of the parasite at time of slaughter. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to *T. spiralis* has been developed but there is a 4- to 5-week period between initial infection in the pig and the presence of detectable antibodies in the animal's blood.[69]

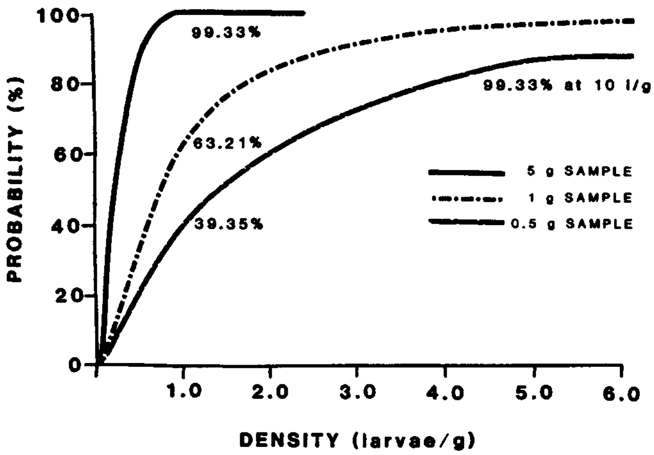

Figure 17-6 Influence of Sample Size and Trichinae Density on Probability of Detecting the Larvae. *Source:* Reprinted with permission from A.W. Kotula et al., Distribution of *Trichinella Spiralis* in the Diaphragm of Experimentally Infected Swine, *Journal of Food Protection*, Vol. 51, pp. 691–695, © 1988, International Association of Milk, Food and Environmental Sanitarians, Inc.

The typical distribution of *T. spiralis* larvae in pig muscles is shown in Table 17-12.[68] The resultant meat from such infected muscles can cause human illness unless the larvae are killed before the meat is consumed. In addition to pork, horse meat has also been implicated as a source of trichinosis in humans.

The state legislature of Illinois passed a law allowing the state Department of Agriculture to inspect

Table 17-12 Distribution of *Trichinella spiralis* in Tissues of Experimentally Infected Swine

Tissue	*Mean Number Larvae g^{-1}*	*Infection Rate, % of Diaphragm*
Tongue	452 ± 51^{a}	116
Diaphragm	391 ± 38^{b}	100
Obliquus abdominis internus (bacon)	130 ± 13^{c}	33
Serratus ventralis (Boston)	116 ± 15^{cd}	30
Psoas major (center loin)	105 ± 13^{cde}	27
Triceps brachii (Boston)	100 ± 18^{cde}	26
Biceps femoris (ham)	83 ± 10^{cdef}	21
Semitendinosus (ham)	74 ± 9^{def}	19
Intercostal (rib)	60 ± 7^{ef}	15
Semimembranosus (ham)	58 ± 8^{ef}	15
Longissimus muscle (center loin)	37 ± 5^{f}	9

^{abcdef}Means (n=31) with different superscripts are significantly different ($p<0.05$).

Source: Reprinted with permission from A.W. Kotula et al., Distribution of Trichinella *Spiralis* Larvae in Selected Muscles and Organs of Experimentally Infected Swine, *Journal of Animal Science*, Vol. 58, pp. 94–98, © 1984, American Society of Animal Science.

pig/swine herds serologically and to seize and destroy infected pigs.[109] An indemnity program compensated the farmer for the loss of the pigs. At present, enabling federal legislation is needed in the US to identify and seize, at the production level, any slaughter animals that have the parasite. Whereas serological testing of the herd at the production unit would be invaluable for identifying farms whose management practices allow cross-contamination of the pigs, testing of every carcass in properly pooled samples at time of slaughter is essential to overcome the potential inaccuracies of the serological tests. Inaccuracies are to be anticipated if (1) the severity of the infection is not adequate to elicit a definitive serological response or (2) the infection is adequate but recent so that the serology does not yet detect the infection.

Toxoplasma gondii

Human infection with *T. gondii* may result from the consumption of meat containing tissue cysts. Slaughter animals, including swine, sheep, goats, and poultry, may be intermediate hosts for *T. gondii*. The organism has not been isolated from beef raised in the US, and even experimentally infected cattle shed the organism from their tissues.[33]

Toxoplasma gondii has been found in pigs in every country where tests have been done. In the US, about 30% of pigs were seropositive for the organism, and of these, about 85% would be expected to have viable cysts in their tissues.[31] In the US, *T. gondii* seropositivity in sheep was 21% to 37%,[32,106] and 15% in horses.[106]

Cats and other Felidae, are the only known hosts for the parasite. Oocysts passed in the feces of infected cats may contaminate feed, water, or soil and thus can be ingested by slaughter animals.[33] The oocysts have been shown to persist in soil for 18 months, even in cold or dry weather.

Meat from infected animals, if not properly cooked, can cause infection in humans. In the US, up to 50% of the population is seropositive for *T. gondii*, whereas in continental Europe and South America, the rate is up to 90%.[34]

Leighty[80] recommended education, treatment of meat, and vaccination of cats as the most logical approaches for the prevention of human toxoplasmosis. Recommended treatments to destroy *T. gondii* in meat include freezing or cooking. Elimination of cats, domestic and feral, from animal production units is one of the simplest methods for the control of *T. gondii* in slaughter animals.

17.5 EFFECT OF STORAGE AND PACKAGING ON THE MICROFLORA OF RAW MEATS

17.5.1 Conditions Used

The spoilage of fresh red meats can result from microbial growth, enzymatic degradation, oxidative changes, and physical deterioration. Larger cuts can be vacuum packaged using oxygen-impermeable plastics. The storage life of chilled pork at 0°C, packaged in different atmospheres was 1.5 weeks in air, 3.0 weeks in high-oxygen modified atmosphere packaging (MAP) (30% CO_2 and up to 70% O_2), 5.0 weeks in low-oxygen MAP (up to 5% O_2), 5.0 weeks in vacuum, and 12 weeks in 100% carbon dioxide.[57] Low O_2 concentrations contribute to the rapid irreversible browning of red meat and therefore are not recommended for palatability reasons.[46]

Fresh red meats for retail sale are typically packaged on a moisture-impermeable tray with a moisture-absorbent pad under the meat, and an oxygen-permeable transparent plastic sheet overlay. Any excess moisture is absorbed by the pad, while oxygen permeability maintains the bright cherry red color due to oxymyoglobin.

The keys to maximizing storage life are to keep the meat only slightly above the freezing temperature, keep it clean, remove essentially all of the oxygen from the package, prevent oxygen from re-entering the package, and provide at least 25% carbon dioxide within the package.[57] Boneless pork primals, packaged in a 100% CO_2-controlled atmosphere, with stringent hygiene and temperature control were transported from the US to Japan; storage temperature was –1°C for unwrapped product and –1.5°C for cartoned product. This pork had a total storage life of 12 to 14 weeks.[57] In contrast, the previous practice of transporting this product gave an average total storage life of 6 weeks.

17.5.2 Spoilage Bacteria

Meat frozen to –40°C is considered to have all of its water in the solid state. At temperatures between –40°C and –2.2°C, variable amounts of water are frozen, depending on the meat composition. At temperatures above –2.2°C, the meat is unfrozen. Frozen meat generally inhibits bacterial growth but does not eliminate it entirely. To extend the storage life of chilled meat, it should be maintained as close to freezing as possible. During storage at –1.7°C for 18 days, the numbers of aerobic bacteria in ground beef increased by about 1 Log_{10} (Figure 17-7).[39] Table 17-13[91] indicates a 4 Log_{10} increase in the total aerobic count and the count of lactic acid bacteria on vacuum packaged beef stored 5 weeks at 2°C compared to 2.7 Log_{10} and 0.2 Log_{10} increase, respectively, at –1°C; after 10 weeks of storage, the difference was less. If one were to consider that –1°C provides 100% of the attainable storage life for chilled pork, then 0°C would be expected to provide 70%, 2°C would provide 50%, and 5°C would provide 30% of the attainable storage life.[57]

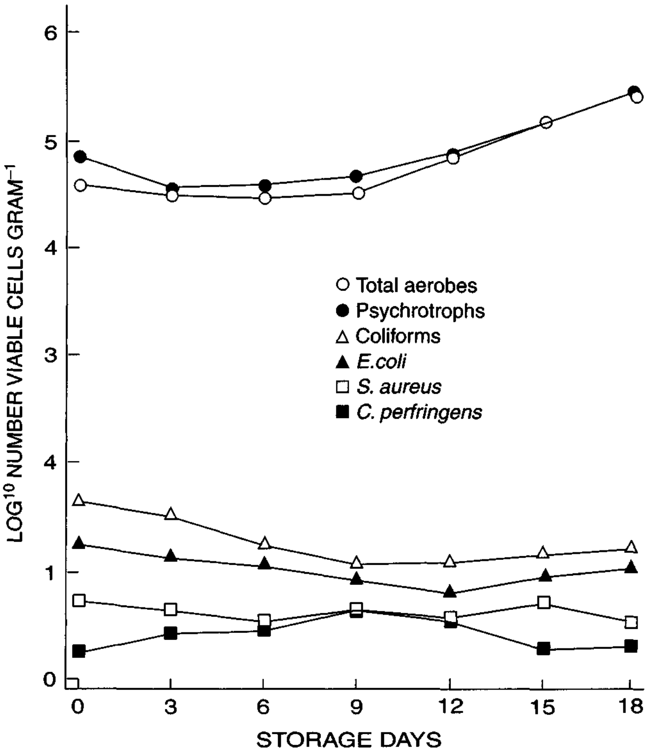

Figure 17-7 Effects of Storage at –1.7 ± 0.6°C for 18 Days on the Microbiological Quality of Ground Beef. Values for storage day 0 are mean Log_{10} counts of 16 samples; all other values are mean Log_{10} counts of 24 samples. *Source:* Reprinted with permission from B.S. Emswiler, C.J. Pierson, and A.W. Kotula, Bacteriological Quality and Shelf Life of Ground Beef, *Applied Environmental Microbiology,* Vol. 31, pp. 826–830, © 1976, American Society for Microbiology.

Vacuum packaging allows carbon dioxide to accumulate; the CO_2 inhibits pseudomonads and allows lactic acid bacteria to become predominant, thus extending the storage life of meat compared with that in air.[22] In such packages, the levels of CO_2 may exceed 20% v/v, whereas the O_2 is depleted to 1% or less. Table 17-13[91] shows the inhibitory effect of CO_2 on *Pseudomonas* spp., *Brochothrix thermosphacta,* and coliforms.

Temperature as well as atmosphere can affect the microbial flora of meat. The flora of fresh pork packaged with 40% CO_2 and 60% N_2 comprised mainly aeromonads, Enterobacteriaceae, and lactic acid bacteria when the meat was stored at 4.4°C, whereas at –1°C the most prevalent bacteria were aeromonads, *B. thermosphacta,* and lactics.[83] At 10°C, Enterobacteriaceae predominate on pork loins in packaging films with oxygen transmission rates of 1 to 26.5 ml $m^{-2}/24$ hours (OTR measured at 23°C, 0% relative humidity, 1 atmosphere pressure).[82] Other researchers found that lactic acid bacteria were the only bacteria found for the first 24 weeks of storage of pork loins at –1.5°C in 100% CO_2. When the loins were exposed to air and put on retail display at 8°C, the pseudomonads proliferated. *Brochothrix thermosphacta* and enterics grew sparingly under these retail conditions.[54] In another study, storage of pork loins in 100% CO_2 at 0°C resulted in a shelf life of about 3 months, and these conditions inhibited the growth of *B. thermosphacta,* Enterobacteriaceae, yeasts, and molds.[8] Vacuum packaging has been found to be generally as effective as any of the other modified atmospheres in extending the storage life.[91]

17.5.3 Potential Human Pathogenic Microorganisms

Chilling meat to temperatures close to –2.2°C inhibits the growth of foodborne pathogens. At 25°C, the typical generation time for psychrotropic bacteria is about 30 minutes; for 20°C, 75 minutes; for

Table 17-13 Microbial Counts (Log_{10} cfu cm^{-2}) on Beef Stored in Different Atmospheres at –1° and 2°C

	Total Count	*Lactic Acid Bacteria*	Pseudomonas *spp.*	Brochothrix thermosphacta	*Coliforms*
Before storage	2.9±0.6	2.1±0.6	2.3±0.8	1.8±0.8	1.0±0.8
After 5 weeks					
–1°C					
1. 100% CO_2	1.7^{a}	1.4^{a}	1.3^{a}	1.1^{a}	0.3^{ab}
2. 50% CO_2/50% N_2	3.5^{b}	2.0^{ab}	1.0^{a}	0.8^{a}	0.2^{ab}
3. 20% CO_2/80% N_2	4.6^{ab}	4.5^{bcde}	1.0^{a}	1.8^{ab}	0.2^{a}
4. 100% N_2	5.4^{cd}	2.4^{abc}	2.5^{ab}	2.0^{abc}	0.9^{ab}
5. Vacuum	4.6^{bc}	2.3^{abc}	2.6^{abc}	3.6^{c}	0.2^{a}
+2°C					
1. 100% CO_2	6.2^{de}	6.2^{e}	1.0^{a}	1.0^{a}	0.2^{a}
2. 50% CO_2/50% N_2	6.5^{de}	4.5^{cde}	1.8^{ab}	1.7^{ab}	0.8^{ab}
3. 20% CO_2/80% N_2	6.9^{e}	5.8^{de}	3.7^{cd}	2.9^{bc}	1.9^{b}
4. 100% N_2	6.8^{e}	3.2^{abcd}	4.3^{d}	3.2^{bc}	1.9^{ab}
5. Vacuum	7.0^{e}	6.1^{e}	4.2^{d}	4.1^{c}	1.6^{ab}
After 10 weeks					
–1°C					
1. 100% CO_2	4.9^{a}	4.8^{a}	1.8^{a}	1.6^{ab}	0.4^{ab}
2. 50% CO_2/50% N_2	6.3^{b}	6.2^{b}	1.2^{a}	1.4^{a}	0.1^{a}
3. 20% CO_2/80% N_2	6.4^{b}	6.2^{b}	2.6^{abc}	2.5^{abc}	0.1^{a}
4. 100% N_2	6.5^{b}	6.4^{b}	2.4^{ab}	3.2^{bc}	0.1^{a}
5. Vacuum	5.9^{b}	5.7^{ab}	3.3^{abcd}	3.9^{c}	0.1^{a}
+2°C					
1. 100% CO_2	6.7^{b}	6.6^{b}	1.4^{a}	1.2^{a}	n.a.
2. 50% CO_2/50% N_2	6.6^{b}	6.3^{b}	4.0^{bcd}	1.4^{a}	0.4^{ab}
3. 20% CO_2/80% N_2	6.4^{b}	6.0^{b}	4.5^{de}	2.8^{abc}	1.9^{b}
4. 100% N_2	6.7^{b}	6.4^{b}	4.7^{e}	3.3^{bc}	1.5^{ab}
5. Vacuum	6.7^{b}	6.4^{b}	4.1^{cde}	3.6^{c}	1.7^{ab}

Note: The initial counts are given as means ± S.D. of five samples. Within counts (means of five samples) and a single storage period means having different superscripts differed at the 5% level (Tukey's test). n.a.=not available.

Source: Reprinted with permission from H. Nissen, O. Sorheim, and R. Dainty, Effects of Vacuum, Modified Atmosphere and Storage Temperature on the Microbial Flora of Packaged Beef, *Food Microbiology*, Vol. 13, pp. 183–191, © 1996, Academic Press, Inc.

15°C, 120 minutes; for 10°C, 200 minutes; and for 5°C it is 1,200 minutes.[104] Scott and Moberg[104] described the minimum growth temperatures of some of the more common pathogens as: *Y. enterocolitica*, –2°C; *L. monocytogenes*, 1°C; enterotoxigenic *E. coli*, 3°C; *A. hydrophila*, 0–5°C; nonproteolytic *C. botulinum*, 3.3°C; *Salmonella*, 7° to 10°C; *B. cereus*, 6° to 10°C; *S. aureus*, 7° to 10°C; proteolytic *C. botulinum*, 10°C; and *C. perfringens*, 12°C.

Although psychrotropic pathogens grow less vigorously at colder temperatures, bacteria cannot be expected to be destroyed by chilling or even freezing. *E. coli* O157:H7 survived 9 months storage at –20°C.[90] *C. jejuni* in ground beef was sensitive to freezing; the number of organisms inoculated into ground beef declined from 6 to 3 Log_{10} after 3 days storage at –15°C, but then remained fairly constant.[110] However, even very low numbers of potentially pathogenic bacteria may have a profound impact on the public health. As few as 500 cfu of *C. jejuni*[102] and fewer than 15 cfu g^{-1} of *E. coli* O157:H7 may cause illness.[30]

17.5.4 Parasites

Parasites, such as *Trichinella spiralis* and *Toxoplasma gondii*, can be killed by freezing. The USDA Certification Program prescribes temperatures and times of frozen storage that should be used to kill *T. spiralis* in pork.[14] These treatments are sufficient to reduce the number of viable encysted larvae of *T. spiralis* by approximately 99% (Table 17-6).[73] The cysts of *Toxoplasma gondii* are inactivated by freezing more readily than encysted *T. spiralis* larvae.[74]

17.6 EFFECT OF COOKING

17.6.1 Spoilage Bacteria

Bacterial contamination of steaks, chops, and roasts occurs mainly on the surface of the meat. During broiling, roasting, or frying, the heat of cooking destroys most vegetative bacteria. For ground beef patties and restructured steaks, however, the possible survival of bacteria in the center of the patty must be considered. Buck et al.[9] reported that raw patties with a calculated thickness of 1.7 cm should be cooked for 6 minutes on each side on a grill at 137°C to reduce total aerobic bacteria from 10^5–10^8 g^{-1} to about 10^2 g^{-1}; reductions were similar for lactose fermenters and *Escherichia coli*.

Coliforms were reduced to less than 1 Log_{10} from initial counts of up to 5 Log_{10}, by cooking 85 g wt beef patties, 9.2 cm in diameter and 1.3 cm thick, on a griddle at 149°C for 2.5 minutes per side or 177°C for 2 minutes or 204°C for 1.5 minutes.[64] The shortest cooking procedure, 1 minute per side at 149°C, reduced numbers of aerobic bacteria by a factor of 10, whereas in patties cooked at 149°C for 4 minutes (well done), 177°C for 3.5 minutes, 204°C for 3 minutes or more, or at 232°C for 2.5 minutes or more, the numbers were reduced by a factor of 10^4 (Figure 17-8).[64] Psychrotropic bacteria were more sensitive to heat than mesophilic bacteria.

17.6.2 Potentially Pathogenic Microorganisms

The US National Advisory Committee on the Microbiological Specifications for Foods recommended 60°C for 8.34 minutes, 62.2°C for 2.11 minutes, 65.6°C for 0.53 minute, or 68.3°C for 0.13 minute for the inactivation of *E. coli* O157:H7 in ground beef.[90] To ensure that consumers will not become ill from the consumption of meat containing potential pathogenic bacteria like *E. coli* O157:H7 or *Salmonella* spp. the FSIS recommends that meat be cooked thoroughly, that is, to 71.2°C (160°F).[45,114] Previously, they suggested meat be cooked until the pink color is no longer present but now they recognize that fat content and other factors can interfere with the use of color as an indicator of degree of doneness. Table 17-14 summarizes the FSIS current recommended cooking endpoint temperatures.[120,130] Ground meat must be cooked more thoroughly than intact muscle because of the occurrence of microorganisms in the center of ground meat patties, whereas the internal portion of steaks and roasts are considered free of bacteria. The minimum cooking endpoint temperature (65°C) is lower for pork cooked to medium doneness in USDA inspected establishments than for pork cooked by consumers (71.2°C) because commercial establishments use standardized cooking procedures.

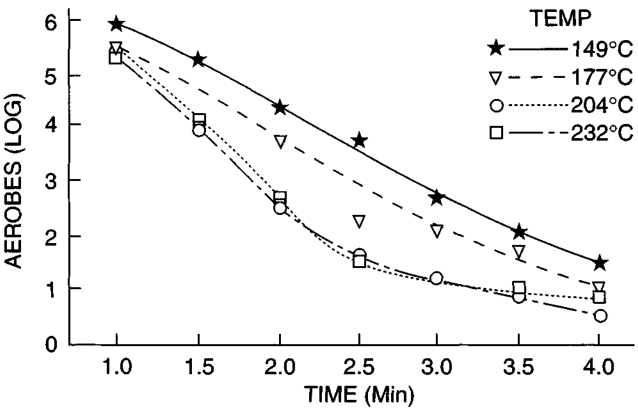

Figure 17-8 Mean Aerobic Bacteria per Gram of Beef Patty, 1.3 cm Thick, After Cooking at Various Temperatures and Times. *Source:* Reprinted with permission from A.W. Kotula et al., Destruction of Bacteria in Beef Patties by Cooking, *Journal of Animal Science*, Vol. 45, pp. 54–58, © 1977, American Society of Animal Science.

17.6.3 Parasites

Initial studies indicated that heating to 55°C was sufficient to destroy the larvae of *Trichinella spiralis*.[94,97] These studies predate some modern cooking methods such as the convection oven, deep fat frying, and microwave ovens. Some of these methods do not allow adequate warmup time to ensure destruction of trichinae at 55° or 58.3°C. Cooking infected pork chops to an endpoint temperature of 66° or 71°C by conventional oven at 162°C, or convection oven at 162°C, or flat grill at 204°C resulted in no detectable viable trichinae.[67] When the chops were cooked to the endpoints of 66° or 71°C by charbroiling or deep fat frying at 162°C, infective larvae were detected. Cooking by microwave is not reliable for the destruction of trichinae as there may be cold spots.[66] The Code of Federal Regulations[14] of the US provides minimum internal temperature and holding times necessary for the inactivation of *T. spiralis* larvae. *T. gondii* larvae are inactivated more readily by heat than *T. spiralis* larvae.

17.7 CONCLUDING REMARKS

At the production unit, animal health and welfare are not only essential for the production of wholesome meat, but are the basis on which sound eco-

Table 17-14 Minimum Internal Cooking Temperatures °C (°F) of Fresh Red Meats[120,130]

	USDA Establishments	*Food Handlers/ Retailers*	*Consumers*
Ground patties (beef, veal, lamb, pork)	70 (157)	70 (157)	71 (160)
Beef, veal, lamb (roasts and steaks)			
Medium rare	63 (145)	63 (145)	63 (145)
Medium			71 (160)
Well done			77 (170)
Pork (roasts, steaks, chops)			
Medium	65 (148)	66 (150)	71 (160)
Well done			77 (170)
Leftovers			74 (165)

nomic decisions should be made. One cannot benefit financially from mistreated or sick animals. Producers of clean, healthy animals should receive a premium price, and those producing contaminated or feeble animals should receive a much lower price. Therefore, animals should be identifiable as to their source. We have discussed the influence of stress during shipping and holding on the predisposition of animals to shed potentially pathogenic bacteria. Standards should be established for the proper handling of livestock during transport and holding prior to slaughter. Financial penalties should be assessed when such standards are not met.

One of the worst enhancers of bacterial growth is extended or poor storage. At the slaughter facility, carcasses and their resultant parts should be systematically shipped out of the facility. Ingredient meats and fabricated cuts should be coded by batch and date so that meats are utilized sequentially, and that problem batches can be easily recalled if necessary. Fresh, unfrozen meat should not be stored "waiting for a better price" or to make a larger load. Shipping distances should be realistic to ensure that the meat arrives in good condition and with adequate shelf life for proper marketing. Trucks and loading docks should be refrigerated and temperature-sensing instruments and abuse disks should be utilized to detect breakdowns in the transport procedures. If fresh meat is being held in the cooler of a meat establishment, a warehouse, a distribution center, or the back room of the meat market, one may logically ask whether it is time that greater attention be given to the shortcomings of the marketing plan rather than attempting to extend the shelf life yet another day or two.

Minimizing the time of holding meat must be used in conjunction with maximal cleanliness and hygiene, and minimal temperatures during all stages of processing, fabrication, and any warehousing.

Regardless of the care taken by the meat industry, some fresh meat will reach the consumer, hotel, restaurant, or institution containing pathogenic bacteria. Therefore, cooking must be relied upon to provide the ultimate safety of the meat. Roasts that have only surface bacteria might be safe to eat less well done than ground beef in which the bacteria must be considered to be present throughout the meat. Methods are needed to be able to measure the internal temperature of hamburgers as they are being cooked on the grill.

Precooked meat is gaining greater acceptance. Current research appears to have solved the greatest deterrent to precooking, namely "warmed-over flavor." Proper cooking of prepackaged meats can provide pasteurized meat of various degrees of doneness that need only to be heated to be ready for consumption. Precooked roast beef is already available in many deli markets and is being used in some restaurants.

Consumer education is a continuing process that can greatly improve the microbiological safety of fresh meats. Every individual must become aware that fresh red meat must be kept clean and cold, and then it must be cooked properly.

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18 Processed Meat Products

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18.1 INTRODUCTION

Processed red meat products no longer have the appearance or perishability of fresh products because their formulation and treatment provides a range of preservation systems. They may be separated into a number of types according to intended storage temperature, ie, ambient, chilled (between 0° and 10°C), or frozen. The different preparation and preservation factors used to give equivalent microbiological stability are determined by the product type and customer usage. Their stability and safety relies on the interaction of their microflora with the nutrients and preservation factors, which can include processing and storage temperatures, water activity (a_w), pH, chemical agents, and packaging. Processing and formulation will kill a fraction of the initial microflora, inhibit or inactivate another part, and allow growth of the remainder.[73] The metabolic activity of the growing fraction can cause spoilage and can limit shelf life, while growth or survival of pathogens may cause safety problems.[227] Because chemical and physical factors alter the appearance, taste, and texture of the product, their use has to be a compromise between the safety and spoilage risk and product acceptability.

Meat and poultry products account for 5% to 10% of consumer spending on food in Europe and the United States (US). They are important vehicles of foodborne illness, particularly salmonellosis, and the economic cost of infections is very high (eg, in England and Wales (1988) the total costs were estimated between £231 and 331 million, of which over 60% was meatborne).[213] The majority of illness originates from raw meat (principally poultry) rather than from processed meat products. In 1996, the US Department of Agriculture (USDA) estimated that contamination of meat and poultry products with salmonella, *Escherichia coli*, campylobacter, and *Listeria monocytogenes* resulted in as many as 4,500 deaths and 5 million cases of illness annually. Associated medical and productivity costs (1993) were estimated at between 4.5 and 7.5 billion US dollars.

Safe, stable product designs can only be achieved within accepted sensory boundaries by identifying the target microorganisms and predicting their fate during processing, storage, and use. An integral part of product and process design is specifying the maximum input of microorganisms that must be inhibited or destroyed. Only when this has been done, can requirements for control, manufacturing and equipment,[7] quality assurance, and storage be defined. This is usually done using a hazard analysis critical control point (HACCP) study.[27,223] Modern techniques, such as time/temperature integrators and mathematical modeling,[148] have been used to assess or predict the impact on product safety and quality of formulation, process, and product handling-linked factors or changes, such as storage conditions or temperature abuse.[124] Combination preservation systems with many components or *hurdles* (Chapter 14) are widely used to minimize the sensory impact of single factors, such as salt or acid. If they were used individually, they would give products with an unacceptable taste.[94,130,132,133]

Despite continuous pressure for milder preservation and extended shelf life, processed meat products have a good microbiological safety record. However, since the 1980s a number of new pathogens such as *Listeria monocytogenes*[67,111,228] and the verocytotoxigenic types of *E. coli* have been associated with meat products.[52] To take account of their higher resistance to some factors, such as cold or acid,[137] product and process designs and manufacturing procedures have been altered to maintain safety standards. The food poisoning problems associated with *L. monocytogenes* contamination in the late 1980s and early 1990s led to a critical worldwide review by the industry of the cumulative impact on product safety of changes in product formulation, processing, and process hygiene. It was concluded that the use of chilled manufacturing facilities provided cooler conditions favoring *L. monocytogenes* rather than lactic acid bacteria or cold-tolerant Enterobacteriaceae that were previously found. Hygiene procedures had not taken account of this, and mildly preserved products containing low levels of a competing flora allowed the growth of *Listeria* species in chill-stored products. The public health impact of *E. coli* O157:H7 outbreaks in the US, Japan, and Scotland during 1996–97 will certainly continue this process of review, especially focusing on hygiene. During the 1990s the industry has increasingly used modern techniques to control the microbiology of products, namely:

1. systematic establishment of realistic microbial heat-resistance values for microorganisms in products
2. HACCP studies to identify "critical control points" in processes
3. targeting of cleaning and disinfection techniques at target microorganisms
4. using mathematical modeling to predict growth and survival
5. analysis and interpretation of the ecological pressures in products and factories to identify conditions selecting for hazardous microorganisms.

Use of these techniques should lead to a reduction in microbiological risks to both producers and consumers.

Where product safety relies on the customer to any significant extent, eg, chilled storage, restricted open shelf life, or thorough cooking, the main safety barrier provided by the manufacturer (apart from ensuring the absence of pathogens in the processed product) is effective labeling, aimed at specifying safe temperatures and restricting the open storage time. Although temperature control during manufacturing and distribution has improved (product temperatures of 5°C or less are not uncommon), it is doubtful whether there have been similar improvements in the domestic part of the products' life cycle. Home use, which includes the open shelf life, has become microbiologically the most risky stage, because the safety of meat products with milder intrinsic preservation systems relies very heavily on effective chill storage. Processing and formulation plus consumer use instructions must be designed to eliminate or control realistic microbiological hazards so that products are free from concern levels of pathogens and toxins and unspoiled at the point of consumption.

This chapter considers the microbiology of nonfermented red meat products (for information on fermented meats see Chapter 19 and for poultry-based products see Chapter 20).

18.2 FACTORS AFFECTING THE MICROBIOLOGY OF MEAT PRODUCTS

The rate of growth and survival of all microorganisms will be determined by the interplay of various factors[161] that are contributed by the product formulation, its processing (ie, procedures that substantially alter the numbers and types of microorganisms present or the environment in the food), packaging, and storage. They can be used by microbiologists to advise the product developer how to kill or inactivate pathogens or control the growth and activity of spoilage microorganisms. These can be classified as follows:

1. ***Intrinsic*** (Properties of meat products). These include the availability of nutrients (eg, extent of cell disruption, residual level of, or addition

of carbohydrates), pH (generally 4.3–7.0), a_w (<0.6–0.99), and the type of humectant (eg, salt, sugar, or glycerol), the presence and concentration of preservatives (eg, nitrite, sulfite, sorbate, benzoate, or parabens) or ingredients with antimicrobial properties, such as spices and smoke constituents. The use and permitted concentration of the latter two groups of substances is governed by law in most countries.[17,115] Interaction of intrinsic factors with temperatures outside the growth range can injure vegetative cells or spores and reduce their viability, as in salt and nitrite in the manufacture of luncheon meat, or enhance the production of inhibitory compounds as by the starter cultures in fermented products. The physical state, structure, and processing of the product will determine the type and distribution of growth. Surface colonies or slime will be found on whole muscle pieces; colonies will be distributed within comminuted or minced products and suspended cells found in liquid suspensions (eg, meat stocks, etc).

2. ***Extrinsic*** (Properties of the environment surrounding the foodstuff). These include process and storage conditions (frozen or sterilization temperatures), gaseous environment, eg, vacuum packaging with high CO_2 and low O_2 concentrations, and relative humidity.
3. ***Implicit*** (The interactions of the microbes—typically interactions with the lactic acid bacteria, associated with fermented products). This can lead to the suppression of some groups or species, by antagonism or competition for nutrients[177,195] or by chemical modification of the environment (eg, acidification or oxygen depletion).

18.2.1 Microorganisms of Public Health Significance

Processed meat products may contain a range of the microorganisms that can cause infection or intoxication of humans. They usually originate from the animal,[203] either forming part of its normal flora or being picked up from the environments it has passed through.[162] The skill of the slaughterhouse workers is an important means of preventing the contamination of muscle with gut contents and fecal matter, which may contain high numbers of infectious and toxigenic pathogens. The input level of microorganisms to products can be minimized by good layout, hygienic operational practices, and rapid chilling, but may be increased by poor hygiene (resulting in contamination) and badly controlled storage conditions (resulting in the growth of contaminants). If carcasses are cooled too slowly, stand for long periods (days), and microbiological contamination of deep muscle or bone has occurred, there is a risk of the growth of mesophilic bacteria, especially salmonella, clostridia, and bacilli, leading to spoilage or infrequently toxigenisis.[107,140] To cause harm, toxigenic pathogens need to grow sufficiently (*ca.* 10^6 g^{-1} or 10^8 cm^{-2} on the surface) for elaboration of toxin to be possible.[109] If there is insufficient cooling and prolonged storage, salmonella or clostridia may grow. Some infectious pathogens are hazardous at such low levels (eg, enteric pathogens such as *E. coli* O157 with a minimum infectious dose [MID] of *ca.* 10 organisms) that their survival is sufficient to be hazardous. *Listeria monocytogenes,* with a MID of *ca.* 100 g^{-1} and a low minimum growth temperature 0° to 2°C, may grow to high numbers in manufacturing areas and products held under chilled conditions,[25] thereby representing a hazard.

Many of the requirements of legislation and handling practices in slaughterhouses and refrigerated stores are designed to maximize the commercial quality and shelf life of meat for direct retail sale. Meat used in the manufacture of meat products requires a higher microbiological quality if processing technologies, such as drying with mild heat (or fermentation) are used as these have a limited ability to reduce levels of pathogens in the product. This requirement is most important in products intended to be eaten without cooking (for example, dry-cured hams, salamis, and raw meat spreads, such as Teewursts).

During manufacture and storage, a factory can introduce contaminants selected by its materials, processes, layout, hygiene (cleaning and disinfection systems), and operating procedures, especially times and temperatures.[15,58] The presence of indicator organisms, such as Enterobacteriaceae, may be used to provide information on the effectiveness of hygiene measures and heat processes. However, it is important that the correct analytical methodology be used to recover injured cells, and that the effectiveness of processing is not overestimated.[19,24] Rapid methods are being developed for the detection of pathogens in meat.[16,172]

Where products contain viable microorganisms, storage temperature and usage after manufacture will play a key role in determining their numbers at consumption. If products are not contained in a primary packaging able to prevent recontamination (for example, in multiportion packs), then storage hygiene plays an important additional role in determining safety and keepability.[86,142,143,222,234]

Infectious Pathogens

The most important infectious pathogens associated with red meat products are salmonellas,[112,178] verocytotoxigenic *E. coli* (VTEC),[34] and *Yersinia enterocolitica*. The Gram-positive, psychrotrophic pathogen *L. monocytogenes* is also an important pathogen.[85]

Salmonella is frequently associated with poultry and pork (detectable on 12% to >50% of carcasses) and less frequently associated with beef raw materials (2% to 5%). It has a low, but variable, infectious dose. Where the fattiness of the product protects the organism from gastric acids, this may be as low as 1 g^{-1} of product consumed. It is facultatively anaerobic and not inhibited directly by vacuum packaging. In meat products its minimum growth temperature is *ca.* 8° to 12°C, with a very extended lag phase; slow growth rates and increased sensitivity to intrinsic preservation factors occur at temperatures below 10° to 15°C. It can grow in mildly acidified meat products, such as hams, pates, and pies (pH 5.0–6.0) and survive at pH 4.4 (lactic acid) and in dried foods (such as cooked meat powder or flakes) for long periods. Survival under adverse conditions is often better at chilled (<10°C) than at higher temperatures, and hence chilling offers little protection against survival of infectious pathogens. It is inhibited by low a_w (<0.96) or *ca.* 8% s/w with 160 ppm nitrite (input level) at pH 6. The extent of survival or killing is dependent on drying conditions, pH,[138] and presence of organic acids, especially lactic acid.[12] As the mechanism of killing is by metabolic exhaustion, it can be killed by a combination of rapid acidification (pH <5.3 within 3 days), the reduction of a_w (≤0.94: >*ca.* 12% s/w), and storage for long periods (>21 days for a 4 log reduction) at temperatures above 15°C, as in the production of salamis. The cells are not heat resistant and are rapidly destroyed at the temperatures used for the cooking of meat products (typical D-value 0.04 minutes at 65°C). Heat resistance can be substantially greater at low (<0.94) a_w[178] and for some types, eg, *Salmonella senftenburg* ($D_{65°C}$ = >1.5 minutes). For further information on salmonella see Chapter 45.

Verocytotoxigenic *E. coli*, in particular *E. coli* O157:H7, are a major concern.[215] They may well be present in raw meats, (such as beef and lamb); the incidence in cattle in Western Europe and the US is very variable, eg, 0.9% to 8.2% of cattle in the United Kingdom (UK) surveys,[125] and is generally increasing for reasons that are not understood, but agricultural practices and better detection methods probably play a role. All are relatively heat sensitive (D_{60} = 0.37–0.55 minutes, z = 7.5°C), therefore reductions in excess of 6 logs are normally caused by cooking (ie, heating to temperatures usually in the range 65° to 70°C). Product composition, especially high fat level (>20% to 30%), reduces the lethality of heating.[1] Their presence in heat-processed meat products indicates inadequate processing (because of incorrect process design or operation/control) or post-process contamination.

There is conflicting evidence of their heat, salt, and acid resistance compared to other *E. coli*. Over the temperature range 10° to 35°C, they have been found to be more tolerant of salt, acid, and nitrite than salmonella, which is the traditional target microorganism. Resistance has been linked to an rpoS regulated protein, whose presence promotes survival (in foods) by offering protection against heat, salt, and acid challenges.[35] There is also some evidence that pre-exposure to stress, for example, an acidic environment, may increase resistance to that factor.[137] This feature has also been found in *Salmonella typhimurium*,[138] and enteropathogenic *E. coli.* For further information on *E. coli* see Chapter 43.

Yersinia enterocolitica is a member of the Enterobacteriaceae, of which some serotypes are pathogenic to humans. These have been isolated from chilled pork, beef, and poultry products, especially if they have an unusually high (>5.9) pH. They are heat sensitive and can remain viable for long periods in acid or low a_w products, high CO_2 (10% v/v) packs, or at chill temperatures (4°C). Therefore, it is important to ensure that not only growth but also survival is minimized by the formulation and processing/storage conditions used for ready-to-eat foods, especially prevention of cross-contamination. For further information on yersinias see Chapter 49.

Listeria monocytogenes is a common contaminant of meat and poultry.[111,151] Its minimum growth temperature is 0° to 2°C (typical lag periods 3 to 30 days and doubling time *ca.* 100 hours). This allows it to become established in cool, damp manufacturing environments, such as on meat processing equipment and in chill rooms.[234] It is able to survive for long periods (*ca.* 100 days) in the presence of high concentrations of salt (10% to 30% s/w) under chilled conditions. The increase in incidence of *L. monocytogenes*[151,228] has probably resulted from the cumulative effects of small changes in processing, which altered a particular ecological niche sufficiently to replace one type of microorganism, eg, lactic acid bacteria, with another closely related one, eg, *L. monocytogenes*. Prolonged chilled storage allows good growth in many meat products.[91] Many current quality assurance procedures are specifically intended to reduce its incidence in processed meat products.[21] They involve pinpointing likely sources of contamination, eg, places likely to support listeria growth, such as slicers, heat exchangers in chill rooms, and conveyor belts, and then using cleaning and disinfecting agents (such as amphoteric surface-active agents), which are effective against listeria.

The factors leading to its prevalence in meat processing areas also allow it to grow in mildly preserved meat products, eg, it grew at 4°C in a meat product with pH *ca.* 5.5 containing 125 ppm nitrite or 0.5% w/v phosphate.[29] It can compete with and may outgrow spoilage microorganisms, such as lactic acid bacteria with minimum growth temperatures *ca.* 8°C, eg, in pate stored at 8° to 10°C.[85,150] It can grow at pH 4.4–4.6 and in some high-salt products (10% sodium chloride on water), even at chill temperatures. Preservatives that have proved effective inhibitors include sodium lactate (4%), potassium sorbate (0.3%), and nisin (400 IU ml^{-1}).[29] Lactic acid bacteria may produce natural antimicrobial compounds that are active against it and other pathogens.[5] *Listeria monocytogenes* is not heat resistant (typical D_{70} = 8 to 16 seconds, z = 7.5°C); a current UK recommendation of heating to 70°C for 2 minutes (or an equivalent heat treatment) is sufficient to ensure its elimination from foods. There is some evidence that processing treatments involving sublethal stress can increase heat resistance.[69] Because only a small part of the population is sensitive to infection by *L. monocytogenes*, there is considerable debate on suitable limits for manufacturing specifications. For further information on *L. monocytogenes* see Chapter 44.

Toxigenic Pathogens

These microorganisms, with the exception of *Clostridium perfringens*, are hazardous because if they grow to high numbers (*ca.* 10^6 g^{-1}),they may produce toxins in meat products; *C. perfringens* produces its toxin in the gut. Toxins may be preformed by growth in raw materials and are not destroyed by freezing. Under certain conditions, toxigenic species may grow without toxin production, but processed foods in which it has grown to high numbers and then been destroyed may cause food poisoning because of the persistence of the toxins. For instance, staphylococcal enterotoxin is very resistant to heating (may survive at 120°C for 10 to 20 minutes), whereas the organism is heat sensitive (typical D_{60} = *ca.* 1 to 5 minutes).

Staphylococcus aureus grows from 6.5° to 50°C (reported range in meat products 10° to 37°C),[219] from pH 4.5–7 (minimum pH 5 in meat products) and above a_w 0.85 (>19% salt on water) in the absence of other preservation factors. It is a frequent contaminant of meat[203] and may produce 1 or more of 10 types of enterotoxin (SEA – H); strains producing SEA and SED are mainly responsible for human food poisoning. Toxin formation in raw meats has not been detected and it is thought that implicit factors, such as the competing flora, may prevent toxigenisis under otherwise favorable conditions.[156] Contamination by food handlers of mildly preserved processed products, where any competing flora has been eliminated by heating or is inhibited by low a_w, coupled with warm storage conditions is a major cause of staphylococcal food poisoning.[109] Under warm semidry conditions, it may become established on food processing equipment and produce toxin. For further information on *S. aureus* in meat products see Chapters 19, 20, and 47.

Clostridium botulinum Types A, B, and F have been associated with food poisoning caused by meat products.[93] The spores if present in meat are usually at a low frequency and number (*ca.* 1 kg^{-1}). Spores of both proteolytic and nonproteolytic strains of *C. botulinum* will survive pasteurization—Type A and proteolytic Type B (typical $D_{112.8}$ = 0.15 – 1.32 minutes, z = 8°–10°C); Type E (typical D_{80} = 0.33–4.3, z-value 8°–10°C). The nonproteolytic strains (Type E and nonproteolytic strains of Types B and F) can grow during prolonged storage at temperatures >5°C (minimum growth temperature 3.3°C), and at a minimum pH of 5.0, under otherwise optimum growth conditions. These strains present a greater food poisoning hazard than the proteolytic types, as their growth to levels forming hazardous concentrations of toxins may occur in the absence of spoilage. The proteolytic strains (Type A and some Types B and F) can grow over the range 12° to 50°C and down to pH 4.6, and to an a_w of 0.94/0.95 (with salt as the humectant). Psychrotrophic, nonproteolytic strains of *C. botulinum* Type E have only been associated with fish products,[93] but can grow very slowly in mildly heated, lightly preserved (*ca.* 3.5% s/w) meat products at commercial chill storage temperatures,[211] and are therefore a potential hazard. Preservatives such as nitrite and salt will reduce or prevent the growth of *C. botulinum* in a meat product, depending on the concentration present,[184,185,186] and an adverse redox potential (eg, the presence of oxygen throughout the product) and the presence of inhibitory microorganisms (such as lactic acid bacteria) may prevent its growth.[39] The toxin of *C. botulinum* is heat labile (inactivated by 85°C for 5 minutes), but heating should not be relied on as a means of ensuring the safety of products where the growth of *C. botulinum* is suspected. For example, botulism (from Type A toxin) has been caused by the consumption of stew that had been cooked and then stored at ambient temperature for 3 days and reheated. For further information on *C. botulinum* see Chapter 41.

Bacillus cereus is widely distributed in soil and vegetation and may be particularly present in meat products that are seasoned with spices or contain cereal or rice.[84] It only becomes a hazard when the cooking process has eliminated competitive species, but left viable spores of *Bacillus cereus* (typical D_{100} = 5 minutes, z = 10°C). Decreasing pH and increasing NaCl and sodium nitrite levels increases the lag phase and extends generation times.[13] For further information on *B. cereus* see Chapter 39.

Clostridium perfringens Type A is ubiquitous in meat products and characterized by a relatively high minimum growth temperature (*ca.* 12°C) and salt tolerance (6% s/w).[183] Because it can grow and sporulate rapidly in meat products at temperatures in the 26° to 51°C range, it is a hazard in cooked meat products that cool slowly because of large containers, or product-unit sizes, or the use of inefficient chills.[41] Growth of *Clostridium perfringens* has been observed in meat soup, sausages, and meatballs.[146,147] For further information on *C. perfringens* see Chapter 42.

18.2.2 Microorganisms of Spoilage Significance

Microbial spoilage of meat is mainly due to the aerobic or anaerobic growth of bacteria. The species involved include *obligate aerobes* (*Pseudomonas, Acinetobacter/Moraxella, Aeromonas, Alteromonas*); *facultative anaerobes* (*Alcaligenes*, Enterobacteriaceae and coliforms, *homo- and hetero-fermentative* lactic acid bacteria including *Leuconostoc, Pediococcus,* and the *Lactobacillus sake/curvatus* group[57,118] and streptobacteria); *Brochothrix thermosphacta*[90]; micrococcaceae, pediococci, enterococci,[49] and *Bacillus* species; and *obligate anaerobes,* such as clostridia. Yeasts and molds (*Geotrichum, Thamnidium,* and *Cladosporium*) may also cause spoilage.

Spoilage microorganisms are commercially significant in meat products when their numbers reach around 10^7 g^{-1}, resulting in sensory changes limiting acceptability and shelf life.[107] Metabolism during and after growth ceases causes changes to taste, odor (eg, sour or ammoniacal), or appearance (eg, gas production or color change—loss of the red color associated with meat or cured meat pigments and replacement with brown, gray, or green). In some extreme cases, the texture may also be altered, by the reduction of pH (caused by acid production) altering the water-holding capacity of the meat, the production of slime, and eventually the breakdown of structural proteins. Because processed meat products contain protein and fat, and in some cases cereal ingredients, the impact of each ingredient on the nature and activity of the spoilage flora is important in determining spoilage. The main changes are as follows: the formation of organic acids and other metabolites, including slime, from carbohydrate breakdown; lipolytic activity; breakdown of complex proteins, peptones, and polypeptides; and production of carbon dioxide and hydrogen peroxide.

Under chilled (4° to 10°C), aerobic storage conditions the surfaces of unpreserved meat products are usually spoiled by the aerobic, psychrotrophic "non-pigmented" pseudomonads, such as the *Pseudomonas-Alcaligenes-Acinetobacter-Moraxella* group, *Kurthia*, or the coryneforms.[217] Their main metabolic products are CO_2 from carbohydrate metabolism, but rapid surface growth can lead to localized carbohydrate depletion and a switch to the metabolism of nitrogenous compounds producing a pH rise and putrid spoilage from amine production. Even under these conditions, carbohydrate metabolism can continue below the surface, and at low oxygen tensions, causing a pH drop because of lactic acid production. If the surfaces dry out during storage (the minimum a_w for the growth of the aerobic psychrotrophs is *ca.* 0.95), growth of the lactic acid bacteria (especially lactobacilli or leuconostocs[57] or *B. thermosphacta*) can cause distinctive types of spoilage. It also occurs if the availability of O_2 is restricted and the level of CO_2 increased, as in a vacuum pack, or if the spoilage flora is distributed below the surface so that the supply of O_2 by diffusion is limited, as in comminuted products like burgers. In the absence of growth of lactic acid bacteria, which reduce the pH, *Bacillus* species including proteolytic types may grow after prolonged storage.[61] If anaerobic conditions develop at high pH (>5.6), then there is growth of the facultatively anaerobic Gram-negative bacteria and putrid spoilage results; they are suppressed when the pH is below 5.4–5.9. If the a_w is below 0.98, the Gram-negative bacteria are progressively replaced by the Gram-positive species, such as lactobacilli, enterococci, and streptococci,[49] *B. thermosphacta*, coryneforms, or *Kurthia zopfii*. Eventually, when the a_w drops below 0.93, bacterial spoilage becomes limited to the micrococci (which are salt-tolerant strict aerobes) and then yeasts and molds become the main spoilage agents.

Many preservation systems use selective factors to steer the type of spoilage or suppress the growth of particular microbial types that cause unpleasant changes.[130] For example, lactic acid bacteria cause the normally acceptable souring of ham and other lightly cured meat products. They are selected by salt level, nitrite, and vacuum packaging and slowly metabolize carbohydrates to acidic products. The drop in pH and accumulation of organic acids (mainly lactic acid, to levels *ca.* 0.5%) prevents putrid spoilage resulting from the ammoniacal products of microbial protein and peptide degradation. Many biochemical methods are available for determining spoilage of meat.[152] Some species of lactic acid bacteria may additionally produce bacteriocins, which are inhibitory to other species.[5]

18.3 PRODUCTION OF MEAT PRODUCTS

18.3.1 Raw Materials Control

Meat products require raw materials of good microbiological quality, but even so they may be contaminated with pathogenic microorganisms, such as salmonellas or *Staphylococcus aureus*.[203] This is especially important if processes have a limited ability to reduce microbial numbers. For example, salami processes typically cause a maximum of 4 log reductions in numbers of salmonella, and the heat processes used for luncheon meats only produce a stable product with an input level of bacterial spores of below 1,000 g^{-1}.[173] Hygiene precautions and the rate of refrigeration will affect the level of contamination and frozen storage can halt growth. There is little evidence that the activity of enzymes of animal origin causes deterioration in frozen meat products, even though they may be concentrated in the unfrozen water and have a long period, typically >1 year, for activity. Most deterioration is caused by chemical oxidation and desiccation. Growth of psychrotrophic and psychrophilic spoilage microorganisms may occur during cooling and chill storage depending on temperature; at below –6°C growth will be halted. Sometimes meat raw materials are stored vacuum packaged or under a modified atmosphere to slow growth and prolong shelf life. The initial pH of such meat and the O_2 permeability of the packaging film are critical to development of the spoilage flora and hence the type of spoilage found.[87] Below 10°C, meat with a pH in the normal range (5.5–5.8) will support the growth of lactic acid bacteria and eventually becomes sour. Meat with pH values >5.8, especially when packaged in films providing a poor O_2 barrier, supports the growth of *Alteromonas putrefaciens* and *Enterobacter liquefaciens*, which produce H_2S and cause greening of the meat.[163]

Some manufacturing technologies may involve lengthy periods of preprocessing before there is any process step that inhibits or reduces numbers of microorganisms. The breakdown of structure, by processing, may speed up growth of any contaminants. For example, ham manufacture may involve the tumbling or massaging of meat pieces at low operating temperatures, typically 4° to 20°C, for periods of hours (up to 48 hours) to improve their cohesion and water-binding during cooking. Depending on temperature during this period lactic acid bacteria may grow and reduce the pH, or reduce the oxygen tension to such a low level that curing cannot take place. During such processes, meat oxymyoglobins are not deoxygenated to give myoglobin (which forms a stable nitroso-pigment), but are reduced to metmyoglobin at the low pO_2, resulting from microbial consumption of oxygen. Metmyoglobin forms an unstable nitroso-pigment[128] and causes color defects in hams. Microbial production of hydrogen peroxide can oxidize meat pigments, also preventing the formation of a stable nitroso-pigment, and leading to color defects. Hence, the effects of prehandling and processing become critical if the growth of microorganisms exceeds a level that can be controlled by the formulation or subsequent processing conditions.

Other animal products including liver and offals, fat, blood, hide, and mechanically recovered meat (MRM) may be used as raw materials.[220] Because of their low commercial value, the bones yielding MRM may not have been rapidly chilled. This coupled with disruption of the structure of the tissue and mixing of contaminants during separation provides an opportunity for microbial growth and spoilage to occur before processing. This is in contrast to muscle where the interior is usually sterile, spoilage is confined to the outside, and is relatively slow. The breakdown of structure by processing may give a higher surface area-volume ratio in products such as MRM or liquids and speed up growth of any contaminants, promoting rapid spoilage. Hence, meat products may be more heavily contaminated with spoilage and pathogenic bacteria (eg, *Staphylococcus*

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aureus, clostridia, and Enterobacteriaceae) than chilled "intact" muscle. Because of higher temperatures caused by the high energy (pressure or abrasion) processing, equipment designed to remove flesh from the surfaces of bones, equipment hygiene and its maintenance, and the initial microbiological quality of the raw material, especially its temperature and storage period, needs to be carefully controlled. MRM is usually stored frozen until it is used. Downstream, manufacturing conditions should be designed to take account of the higher concentration of microorganisms it can introduce. Therefore, these materials should be considered less stable and their shelf lives should not be based on those achieved under similar conditions by conventionally butchered muscle tissue. Nonmuscle materials may exhibit typical forms of spoilage, for example, hemolytic spoilage may be associated with liver and kidneys and lipolytic spoilage with hides and fatty tissue.

18.3.2 Process Control

Pathogenic microorganisms and/or their toxins may be present in raw materials or introduced during processing, storage, or usage; realistic incidence and amounts should be identified using a risk assessment.[27] The means available for control should be based on the use of HACCP to identify the Critical Control Points in the process.[199] Predictive models for growth, survival, or killing of the major spoilage organisms and pathogens may be used for risk assessment. Its output and the HACCP study should be used to determine the design and the operational targets or limits for the manufacturing process. A HACCP study will only be effective if a manufacturing plant operates against a background of Good Manufacturing Practice (GMP) such as outlined in Codex,[37] in the European Union (EU) Regulations,[63] or the US Federal Register.[73] Topics typically covered by GMP guides for the meat industry include the following:

1. Minimum requirements for the manufacturing environment, especially layout to prevent cross- or recontamination of in-process material or product with pathogens or spoilage microorganisms
2. The hygienic design and construction of buildings, stores, and equipment
3. Process operation, control and monitoring
4. Lot traceability
5. Maintenance of buildings and equipment
6. Sanitation, hygiene, and pest control
7. Personnel, skills, and competencies

The Critical Control Points (CCPs) are those additional process activities or constraints on formulation, packaging, storage, or usage that must be applied to ensure safety (and keepability). These may include the following:

1. Control of temperature and time (eg, cooking, sterilization, cooling, and storage conditions)
2. Activities and conditions providing the intrinsic properties of the product relevant to the preservation system (eg, acidification, reduction of a_w, or addition of chemical preservatives such as nitrite or sulfite)
3. Packaging, seal integrity, and physical and gas barrier properties of the primary pack; correct labeling may be a CCP if the consumer is involved in the safety chain (ie, for cooking or use of controlled (frozen or chilled) storage conditions)
4. Product unit or batch physical dimensions
5. Storage conditions.

18.4 PRODUCT TYPES

18.4.1 Uncooked, Processed Meat Products

Typical products are fresh sausages and burgers, which may be stored frozen or chilled and are intended for cooking by the customer. British fresh sausages are made from finely chopped and mixed meat, fat, rind, herbs and spices, and a cereal (carbohydrate) component—rusk. They contain sodium metabisulfite as a preservative and are stuffed into edible casings made from collagen or sheep gut. Burgers are made from more coarsely chopped meat, usually they do not contain nonmeat ingredients. They contain salt to aid binding of the meat particles to one another and are molded into the typical patty shape using pressure. The general manufacturing process for fresh sausage and burgers is shown in Table 18-1.

The manufacturing processes do not cause any reduction, and may even increase, microbial numbers if processing conditions are too warm and hygiene is poor. Immediately after manufacture, these products will contain any spoilage (such as Gram-negative aerobic bacteria, lactic acid bacteria, and Enterobacteriaceae) and pathogenic microorganisms (such as salmonella, *L. monocytogenes*, verocytotoxigenic *E. coli*, *Campylobacter* spp., or *Y. enterocolitica*), derived from the meat raw materials. During storage, temperature and access of oxygen play a major role

Table 18-1 Manufacturing Process for Burgers and Sausages

Typical Process Step	*Microbiological Importance*
Selection and preblending of raw meat and fat materials	Concentration and types of microorganisms in raw materials
Mincing or flaking	
Mixing with ingredients, eg, rusk and sulfite (sausages) or onion (burgers)	Preservative effect of sulfite, nutrient effect of carbohydrate, time and temperature (typical total process duration usually <8 hours and <12°C)
Forming, eg, patty shape (burgers) or stuffing into casings (sausages)	
Storage, frozen (≤–12°C for up to 1 year) or chilled sausages (≤10°–15°C for 1–2 weeks)	
Burgers (<5°–10°C for 7–10 days)	

in determining spoilage and the survival or growth of pathogens.

During chilled storage of British fresh sausages, a facultatively anaerobic spoilage flora develops.[10] It is selected by the preservative sodium metabisulfite (450 ppm maximum input level, controlled by legislation) and some additional factors. These include the presence of carbohydrates from the rusk and the low subsurface oxygen tension resulting from surface growth of aerobes. The flora is numerically dominated by either *Brochothrix thermosphacta* or yeasts[44] growing beneath the surface—depending on the origin and previous storage conditions of the meat.[44,50] Meat from some manufacturers, typically those with very dry, warm chills, may develop a flora containing substantial numbers of lactic acid bacteria. At chilled storage temperatures, sulfite inhibits growth of Enterobacteriaceae[9] especially salmonellas, which are a frequent contaminant[11]; *Campylobacter* species are inhibited by storage conditions.[164] When natural casings are used they may contribute halo-tolerant and enteric bacteria to the storage flora.[180]

In contrast, the spoilage flora of chill-stored burgers, which do not contain preservatives, is similar to that of fresh, aerobically stored meat, unless they are vacuum packed. In both cases, their spoilage flora depends mainly on pH[43] and oxygen tension. During aerobic storage, spoilage is caused by the Gram-negative species, producing CO_2 as an initial end-product and then deamination metabolites. During their typical shelf life (about 7 to 10 days at 5° to 10°C), strict aerobes (such as pseudomonads and acinetobacters) form only a small part (<1%) of the spoilage flora, because microorganisms are distributed relatively uniformly throughout the product and mostly subject to a low pO_2. Cross-sectioning of the product can show the progressive depletion of oxygen beneath the surface by microbial metabolism, as evident from changes in meat pigment color from red to brown; oxidation at low pO_2 gives metmyoglobin, which has a typical brown color.

Temperature determines which microbial contaminants grow; at <12°C salmonellas are inhibited, but *L. monocytogenes* may grow down to 1° to 2°C and other microbes may survive (eg, *Campylobacter*). *Staphylococcus aureus* may produce toxin if low-count meat is stored above about 20°C.[62] For complete inhibition of spoilage species storage below the product freezing point, typically –2° to –6°C, is required.[21] Under deep freeze conditions some species (especially Gram-negative bacteria) may be injured by the freezing process. In practical terms, freeze injury cannot be used to improve the safety of the product, but it is a major influence on the microbiological methods that need to be used to estimate microbial numbers. For an accurate estimate, resuscitation techniques will need to be used.[65,74]

As these products contain raw meat, customer safety depends on cooking to provide an adequate reduction in numbers of infectious pathogens.[77] Product dimensions and cooking instructions should ensure that good quality and a safe product results from use of the recommended cooking conditions. Undercooking will allow the survival of any pathogens present. For example, inadequate deep fat frying caused a hospital outbreak of salmonella food poisoning.[64] Time/temperature requirements may be affected by the storage conditions (eg, temperature at the start of cooking) and product composition.[1] Freezing and fluctuating temperatures inducing stress responses or producing stationary phase cells can also increase microbial heat resistance (eg, *E. coli* O157:H7).[110] Various experimental methods, besides direct temperature measurement (which may be inaccurate if the slowest heating point in the product is not correctly identified), have been developed to determine the endpoint temperatures of products after heating, such as inactivation of lactate dehydrogenase.

Microbiologically Important Features of the Manufacturing and Formulation Technology

The key microbiological mechanisms at work in these products are selection and distribution of the microflora by processing, especially temperatures, cutting and mixing, and the packaging system. The controlled addition of a preservative, such as sodium metabisulfite, and the implicit factors, such as inhibition of Enterobacteriaceae by the growth of lactic acid bacteria and prevention of enterotoxin production by *S. aureus* by the growth of the spoilage flora, are also important. The mincing (or grinding) and mixing processes will disrupt the cellular structure of the meat, liberating cell contents, and dispersing microbial cells more uniformly within the meat matrix.[116] This may affect the apparent microbiological quality of the product as when the variability (standard deviation) of the log mean counts is reduced as a result of the mixing processes (standard deviations are typically reduced from $\pm 1.5 \log_{10}$ to $\pm 0.25 \log_{10}$), and the measured average (mean log) count will increase.[116] This increase may cause detectable numbers of any microorganisms present at low numbers to rise above the detection level of an analytical method, apparently indicating contamination during processing. This effect is exaggerated in mechanically recovered meat (MRM). The microbiological quality of these products will reflect directly the quality of raw materials, including any increase or recontamination during processing and storage. Product developers assigning product shelf lives should always take account of realistic starting levels of spoilage microorganisms, the microbial level at which unacceptable quality loss is caused, and the likely storage conditions, which will dictate microbial growth rate and hence the spoilage rate.

As cooking prior to the consumption of a raw meat product is an essential part of the processing safety chain, minimum cooking time and temperature must be ensured by providing the consumer with validated cooking instructions.[64] There is some debate over the necessary reduction in pathogen numbers that a cooking process should deliver,[170] but a 4 log reduction in infectious pathogens should be considered a minimum, with 6 logs as a target. The effectiveness of any cooking instructions should be validated, for example, by the use of the bio-integrator method.[97] Different types of cooking methods vary in their heating ability. For example, microwave heating does not produce an even or predictable distribution of heat and therefore temperature. Thus, it cannot be relied on to pasteurize products consistently.[181]

In-process controls focused on temperature and hygiene should be backed up by supplier auditing. Specifications should cover the control and monitoring of raw material quality, manufacturing hygiene, storage, and distribution temperatures.

18.4.2 Cooked, Uncured Meat and Meat Products

Typical products are heat-set joints (usually beef, pork, or poultry). A typical shelf life of a cook-in pack at 0° to 3°C is 12 months) but when sliced, a typical shelf life would be 2 to 3 weeks under normal retail chill conditions. As the meat components of ready-to-eat meals, pies, small goods, or savory products, containing offals such as liver or heart the typical shelf life would be 1 week. They may be oven or fryer cooked or produced using *sous vide* technology resulting in an extended storage.[160]

The main microbiological hazards arise from microorganisms surviving the manufacturing heat process or from recontamination after heating. Products may be frozen or stored under chill conditions. Under chill storage conditions, vacuum packaging may be used to inhibit the growth of aerobic species.[102] The type of packaging and packaging atmosphere modify the type of spoilage and may also reduce the risks of pathogen growth during chilled storage.[105] Typical cooking processes will kill vegetative cells, but will not reduce numbers of bacterial spores, which remain a hazard if storage temperatures fall within their growth range. The infectious and toxigenic vegetative pathogens—salmonella, *L. monocytogenes, S. aureus,* and *Y. enterocolitica*—generally do not survive commercial heat processes, unless there is underprocessing. Their presence usually results from recontamination after cooking.[103,104,106] Where marginal heat processes are used, for example, in *sous vide* products, heat-resistant vegetative microorganisms such as streptococci and lactobacilli may survive cooking and cause spoilage.[103] However, spoilage bacteria are far more commonly introduced during processing, especially if there is unhygienic handling of the unwrapped product during cooling, slicing, and packaging. Often contaminants will have been selected, or be well adapted to the environment in the factory and equipment, and hence able to grow in the product. Storage temperatures below about 8°C will inhibit lactic acid bacteria (LAB) but can allow the growth of *Listeria* spp. and psyschrotrophic Enterobacteriaceae, which are often inhibited by growth of LABs at higher temperatures. Lactic acid and lactates have been used to prevent growth at lower temperatures.[98,101] Unusual cold-growing clostridia causing spoilage have been isolated from vacuum packed products, such as cooked pork or beef.[126]

In the *sous vide* process, products are vacuum packaged before cooking to retain their shape and maximize heat transfer; they usually receive mild heat processes in the 65° to 75°C range. Because spores may survive these low heat processes, storage at a low temperature (below 4°C) or for a restricted time at a higher temperature (eg, up to 2 weeks at 10°C) is essential to prevent the growth of psychrotrophic bacteria, especially *C. botulinum*.

In some countries, chemical additives, such as organic acids and their salts, may be included in chill-stored products to slow the growth of pathogens (and spoilage bacteria). However, this approach has met with limited success because of the adverse impact on the taste of bland products. Lactic and citric acid, or sodium acetate (0.5% w/w to give concentrations of *undissociated* acid 0.01% to 0.05%), have been used.[207,232] Also sorbate (0.26%)[214] has been used to improve the stability of products such as low-salt turkey. Lactate (*ca.* 3% w/v) and salts of *para* hydroxy benzoic acid have been used to inhibit *S. typhimurium*, *E. coli*, and *L. monocytogenes* in pork products stored at temperatures between 5° and 20°C.[99,101,206,233] The product applicability and use concentration of these preservatives is limited by sensory acceptability (usually concentrations over 0.5% are organoleptically unacceptable), and permitted additives are specified by law in most countries.

Microbiologically Important Features of the Formulation, Manufacturing, and Packaging Technology

As the products are largely unpreserved, microbiological quality relies on control of temperature and prevention of recontamination; see Salvat et al.[192] for a HACCP study of a typical process for the production of a vacuum packed chill-stored product.

Products are cooked to give characteristic tastes, textures, and colors. From a microbiological point of view minimum coldest point temperatures in the range 65° to 85°C are sufficient to destroy (eg, 6 log reduction) vegetative spoilage and pathogenic bacteria.[66] Accurate measurements of the minimum or integrated heat processes are required because generally the lowest possible heat treatments are used to minimize texture changes and weight losses due to "cook-out" of soluble proteins. It is also important that temperature distributions in heating vessels are determined and product heating times take these into account. Use of indirect indicators of the application of defined heat treatment has been proposed, eg, the use of denaturation of acid phosphatase for determining the extent of heating given to picnic hams.[119] After cooking, high standards of process hygiene, including effective raw/cooked segregation are necessary to prevent cross- and recontamination of products with microorganisms from raw materials or the indigenous flora of the factory. In addition to forward flow, the clean room concept is often applied and includes hygiene control measures such as prohibition of entry and separation of personnel from factory areas handling uncooked material, specified clean clothing, a filtered air supply, and a cool dry environment with good cleaning and disinfection.[22,36,131,204] Where these processes and areas are well managed, packed meat may be so clean that spoilage bacteria are only infrequently detected; a spoilage flora of variable composition can originate from process survivors (eg, spore formers), the factory environment, and any process aids used.[231] A typical process for the manufacture of sliced roast beef is shown in Table 18-2 and for a cooked meat pie in Table 18-3.

Table 18-2 Typical Process for Sliced Roast Beef

Process Steps	*Microbiological Importance*
Selection and butchery of raw meat and fat materials	Concentration and types of microorganisms
Tumbling and massage of prime cuts, introduction of salt and phosphate	pH modification
Trimming	
Packing into clamps or nonedible casings for shaping	Dimensions of pack
Cooking process in steam, water, or air, typically 68°C chamber temperature; >65°C center temperature	Cooks product and eliminates spoilage and pathogenic vegetative bacteria; bacterial spores are unaffected
Cooling in container, <10°–15°C within 4 hours	Under "high hygiene conditions" to prevent recontamination
Unpacking	"High hygiene conditions" to prevent recontamination
Roasting, to provide surface browning—*ca.* 180° to 250°C	May provide surface decontamination
Air cooling, <5°C in <12 hours	
Slicing	Hygiene of slicer is mainly responsible for initial flora on product
Vacuum packing	O_2 and CO_2 permeability of packaging film and storage temperature will determine shelf life and type of spoilage
Cooling, <2°–5°C within 8–12 hours	
Storage, <10°C	

Table 18-3 Typical Process for Meat Pies

Process Steps	*Microbiological Importance*
Selection and butchery of raw meat and fat materials	Concentration and types of microorganisms
Tumbling and massage of cuts	Introduction of salt and phosphate; pH modification
Trimming and size reduction	
Mixing, seasoning, and heating of meat mix	Gelling of starch and thawing of frozen meat pieces
Cooking process, typically 100°C for some minutes	Kills vegetative cells and some spores
Meat mix cooling in container	
Indirect cooling to 10°–35°C within 4 hours	Under conditions to prevent recontamination
Dough preparation	Use of scrap dough must be limited to prevent the use of "sour" dough
Dough blocking and resting to make cases	
Filling and lidding of pie cases	
Baking of filled pies, center temperature of product >90°C	Kills vegetative cells and some spores
Air cooling of pies, <2°–5°C within 8–12 hours	High hygiene conditions required to prevent recontamination, especially with molds
Wrapping	Moisture-permeable wrapping used to keep crisp crust and may prevent recontamination
Storage <10°C	To prevent growth of spores and other contaminants

To minimize the chances of surviving spores outgrowing, products should be cooled as quickly as possible after cooking. This must be achieved without freezing the product surface (*typical* maximum cooling times for 2.5 kg joints are <8 hours between 50° and 10°C). Spoilage defects (eg, greening or gas production) or unsafe product may result from the growth of clostridia, such as *C. perfringens*, during slow cooling. This may occur if product units are too large to cool quickly or if inefficient or overloaded cooling equipment is used. After cooling, storage at 5° to 8°C is needed to prevent the growth of psychrotrophic spores, typically *Bacillus* spp., which may cause musty flavors or slime formation after prolonged storage of vacuum packaged products (>10 to 20 days). In EU countries, maximum temperatures during processing meat products are defined by law, eg, in slicing rooms (<12°C, except where a derogation for technical reasons is obtained) and during distribution and retail storage (<7°C). Rapid cooling is also required. If lightly preserved products are cooked and stored at 0° to 2°C in their original primary pack, their shelf life may only be limited by chemical changes (including rancidity and color loss), as recontamination and spore outgrowth are prevented. For most products, shelf life is limited by microbial spoilage or contamination by post-process additions, eg, gelatin and garnishes on pates can further limit shelf life. Such additions may be heat treated to decontaminate them or they may be preserved with acid or salt. The introduction of nondecontaminated material into hygienic areas significantly increases the risks of product recontamination.

Many cooked products are vacuum or modified atmosphere (MA) packaged after slicing or repacking; the shelf life of these is usually less than 28 days under chill storage. Vacuum packaging relies on the ability of the contaminants to remove residual O_2 and produce CO_2, or MA packaging uses gas flushing with N_2, CO_2, or gas mixtures (70% N_2, 20% CO_2, and 10% O_2.) to extend shelf life. The lack of oxygen and the accumulation of CO_2 encourages the growth of the slower-growing acid-producing lactic acid bacteria, and suppresses the growth of the rapidly growing aerobic spoilage flora (typically pseudomonads), which can produce ammoniacal end-products. Therefore, shelf life is limited by the growth and metabolism of LABs. If the pack pH is >5.6, the Enterobacteriaceae can grow and produce putrid spoilage and product blackening from H_2S production.

18.4.3 Raw-Cured Products

The intrinsic preservation feature that gives these products, which are usually pork based, their unique identity is the addition of nitrite and salt, and in some cases air drying. Nitrite and salt react with meat even without heat, giving stable red colors, flavors, and textures.[205] The range of raw-cured products may be subdivided according to a_w and product usage as follows:

1. High a_w products intended for refrigerated storage and cooking, eg, bacon (typical shelf life 3 to 6 weeks)
2. High a_w products for refrigerated storage and raw consumption, for example Teewurst (a minced, raw pork product, typical shelf life up to 1 week)

3. Low a_w (eg, below 0.8) products such as ambient-stable raw, heavily salted, dried hams (eg, prosciutto), bacon, and nonfermented dried sausages (typical shelf life 6 to 12 months)

In EU countries, the maximum legal input level of sodium or potassium nitrite is 150 ppm; an equal amount of nitrate may also be added. Current input levels result in residual levels from 0–50 ppm after storage. In some countries, to prevent excessive addition, they may only be added premixed with sodium chloride. Nitrite addition is controlled because during high temperature cooking (eg, frying of bacon) nitrosamines may be formed by the reaction of nitrite with amines in foodstuffs. Phosphates may be added, principally to enhance water retention, and ascorbate is used to enhance color formation.

Nitrite has wide-ranging antimicrobial activity, inhibiting many sporing and nonsporing microorganisms (see Chapter 19).[14,20,28] It selects for lactic acid bacteria and yeasts, which eventually cause spoilage of these products; yeasts are associated with the spoilage of low a_w products. Its antimicrobial activity is pH dependent and increases as pH decreases (from pH 7 down to 5); nitrous acid is thought to be the inhibitory molecule.[33] Low water activity (*typical range* 0.99 to 0.9) achieved by salt addition (typical salt/water levels are 2.5% to 15%) or drying enhance the preservative effect. The use of multiple factors to control growth is called Hurdle Technology (see Chapter 14).[130,132] Typical spoilage species can grow in products and in-process materials containing nitrite at the normal input levels (150 ppm), in up to 10% NaCl, at high CO_2 (20%) concentrations, and down to 8°C. Many types can produce slime (dextran), for example, in curing brines or on sliced products, and in the presence of O_2, *Lactobacillus viridescens* and *Streptococcus faecium* can cause greening of raw products by oxidation of meat pigments (by H_2O_2 formation). The Gram-negative bacteria typically associated with raw meat are inhibited by nitrite (input level 150–200 ppm) or if the pH of the meat is below about 6.

Nitrite is active against *C. botulinum*; particularly in the presence of sodium chloride.[38,182,187] It is also effective against *C. perfringens* and the cold-growing clostridia.[8,107,183] Toxigenic types of *C. botulinum* differ widely in their resistance to NaCl and NO_2. Type A and proteolytic Types B and F are more resistant than Type E and the nonproteolytic Type B and F strains (see Chapter 41). At 20°C, 10^6 spores of *C. botulinum* Type A and B sample did not produce toxin in nitrite-free bacon, whereas at 30°C, 10^4 spores grew to produce toxin in nitrite-containing bacon.[38] The precise mechanism of action of nitrite on microorganisms in meat products is unknown. It may inhibit by reacting with either amino groups, sulfydryl compounds, or with the pyruvate-ferredoxin oxidoreductase in the phosphoroclastic reaction; the latter reaction is used during the initial stages of spore outgrowth.

High a_w *Products*

In bacon, the low salt level (now generally below 3% salt-on-water [s/w]) and slightly acidic pH (pH 5–6) determine the type of microbial growth and spoilage. The use of oxygen-impermeable films with MA packing slows the loss of color and the growth of the aerobic spoilage flora, promoting the growth of salt-tolerant facultatively anaerobic species such as streptococci, leuconostocs, pediococci, lactobacilli, and some vibrios.[79] Depending on the temperature, pH, and a_w, the microflora may exhibit lipolytic activity against triglycerides or proteolytic activity against peptides and proteins or form hydrogen peroxide and organic acids and other metabolites (eg, dextrans and acetoin) from carbohydrates. The streptococci, micrococci, lactobacilli, and leuconostocs dominate the spoilage flora of low-salt bacon and similar products with pH values below 5.4.[229] If the pH is above 5.5–5.6, then the facultatively anaerobic Enterobacteriaceae take over and their growth may cause sulfide spoilage (blackening). In 1982, Gardner[78] reported that vacuum packed low-salt bacon (<7% s/w) soured at below 20°C because of the action of lactic acid bacteria; at 30°C, growth of Enterobacteriaceae caused it to become putrid. During the storage of high-salt bacon (*ca.* 10% s/w) at 20° to 30°C, the micrococci, leuconostocs, and coagulase-negative staphylococci grew and produced a cheesy spoilage. Since 1982, salt levels have dropped and 2% to 3% s/w is now common. If salt concentrations are maintained at 3% to 4% s/w, low input levels of nitrite (50 ppm) may be sufficient for the technological needs (color retained and spoilage inhibited) provided the bacon is stored below 10°C.

Because salt levels are low in commercial bacon, microbial growth is largely controlled by temperatures during curing, processing, storage, and distribution. Vacuum packaging of the sliced product extends the shelf life to 3 weeks or more at 3° to 10°C by selecting the spoilage flora and slowing its growth. The O_2 permeability of the film used plays a major role in determining the composition of the spoilage flora and the shelf life; the lower the O_2 and CO_2 permeability the longer the shelf life. In vacuum packs, the spoilage flora grows more quickly on the "fat" (micrococci, streptococci, and lactic acid bacteria—lactobacilli, pediococci, and leuconostocs) than on the "lean" (mainly lactobacilli). But it is growth on the lean, accompanied by the breakdown products of protein/peptide metabolism that leads to spoilage; higher salt levels favor growth of the leuconostocs, which have weak proteolytic activity. Breakdown of the cured pigments by microbial action gives green or brown colors, usually prior to slime formation, and is the typical form of spoilage. The assurance of minimum customer cooking time and temperature by provision of microbiologically validated cooking instructions is essential for product safety.

Low a_w *Products*

The low a_w of these products, such as dried hams and sausages, selects for the more a_w-resistant types of lactobacilli, micrococci, microbacteria, enterococci, leuconostocs, and yeast and molds. Depending on the preservation system, products may be chill stored (typical closed-pack shelf life 7 to >200 days at 7° to 10°C) or be shelf stable. All have typical microfloras, which develop during storage; differences are largely determined by a_w and packaging. Dry-cured ham stored in air supports the growth of micrococci[209] and yeasts. The survival of infectious pathogens, such as salmonella, verocytotoxic *E. coli*, and *L. monocytogenes* in these products is the major microbiological safety hazard,[196-198] and may lead to illness.[30] In dry-cured hams, the formation of staphylococcal enterotoxin has not been detected in products with an a_w of <0.89 at 35°C but was found in a product with an a_w of 0.92 at 20°C.[226] Enterotoxin production in these products has been found when the competing flora is suppressed, for example, by surface scalding followed by contamination with *S. aureus* by human handling. Smoking may also be part of the preservation process, carried out as part of surface drying and leading to deposition of anti-oxidant and antimicrobial compounds on the product surface.[18] The type of wood used to provide smoke and the smoke-generating equipment used must be carefully controlled to minimize the chances of carcinogen production during processing. After drying a high standard of hygiene during handling and slicing is essential to prevent contamination with a microflora well adapted to growing on the product, and within the pack, and capable of causing premature spoilage. The primary packaging materials have two functions, to limit the chances of product recontamination after processing and to limit the uptake of moisture during storage. Where vacuum or MA packing is used, the films used should be able to retain a nitrogen gas atmosphere, rich in CO_2, to reduce the growth rate of, or suppress, aerobic spoilage microorganisms, especially molds.[129] High CO_2 (20%) atmospheres may be used in gas-flushed packs for sliced products, to prevent the slice adhesion caused by vacuum packaging, but choice of a packaging film with low O_2 permeability is always essential to retain quality and prevent rancidity.[165] Although this packaging is mainly done to preserve the appearance and taste of products, it may also be done to minimize the chances of mycotoxin formation, because a high percentage (78%) of molds from these products have proven to be potentially toxigenic.[135]

Microbiologically Important Features of the Manufacturing and Formulation Technology

Products may be whole joints, such as legs, or prepared from muscle pieces, minced, or comminuted meat. Contaminants remain predominantly on the outside of whole joints, especially if dry curing is used. They may be carried into the muscle by butchery, within bone, or redistributed by comminution or by liquid-curing equipment, especially by the recycling of "injection" brines from multineedle or other injection equipment. The rate of curing is dependent on the rate of diffusion and the intramuscular distances involved. If curing salt does not reach the center of the side, especially the "pocket" of the scapula or other deep muscle and reduce the a_w, then bone taint may be caused by growth of contaminants (eg, alcaligenes, proteus, or clostridia) that are normally inhibited by the high salt concentration (7% to 10% s/w). When chilling prior to salting is ineffective, or process times before effective curing are too long, spoilage similar to that of raw meat held under similar conditions may occur. Processing conditions are less severe than are used for traditional dry hams (see below), and there are differences in the salt tolerance of microorganisms growing as a result of undercuring.

High a_w *Products for Cooking (eg, Bacon)*

Bacon may be cured using brine or by the dry application of crystalline salt; a typical process for commercially produced brine-cured bacon is shown in Table 18-4.

The types and number of microorganisms in a curing brine will vary with its salt and nitrite/nitrate content, its age, the hygiene of tanks, etc, and the initial condition of the meat placed in it. A traditional production method of bacon production, Wiltshire cure, involves the immersion of sides of pork and/or pump-

Table 18-4 Typical Process for Low-Salt, Smoked Bacon Manufacture

Typical Process Stages	*Microbiological Importance*
Carcass preparation—stunning, exsanguination, scalding, dehairing, scraping	Contamination of carcass with pathogens
Evisceration and splitting of carcass	Contamination of meat with pathogens
Grading of meat	
Chilling; 4° to 5°C within 12 hours	Prevent growth of mesophilic pathogens
Trimming of sides	
Injection with curing brine, >8% by weight, >18% salt + 0.15% sodium nitrite	Prevent growth of anaerobic pathogens, eg, clostridia
Filling tanks with cover brine (*ca.* 20% salt + 0.15% sodium nitrite)	Allow development of typical microbial association of salt-tolerant bacteria
Holding in tanks at *ca.* 5°C, 2 to 5 days	
Drainage and cooling	
High temperature/short time smoking (60°C for 2 to 4 hours)	Some reduction of surface contaminants
Cooling to 0°C	
Slicing and vacuum packaging	Recontamination with adapted spoilage flora; O_2 and CO_2 permeability of packaging films will determine shelf life
Storage	Temperature will determine shelf life

ing them with brine by injection, followed by draining and maturation. Although brines are usually chilled to preserve them, they can contain very high numbers of vibrios, aciduric lactic acid bacteria, enterococci, and micrococci (especially in brines >3 months old).[78,79] Many of the spoilage species associated with raw meat (eg, pseudomonads) are inhibited and die out at the high level of salt (up to 20%) in brines. Modern bacon curing generally uses clean brines and the flora present on the bacon therefore reflects that of the meat and is numerically less and less diverse. In bacon containing 6% s/w, *S. aureus* produced toxin at 27°C but not at 15°C.[200]

High a_w Products for Raw Consumption (eg, Teewurst)

The manufacture of traditional, spreadable, high a_w products, such as Teewurst, which are eaten raw, may or may not include a short fermentation stage (2 to 3 days), to reduce the pH to *ca.* 5.3–5.5 and produce a typical flavor; a typical manufacturing process is shown in Table 18-5. These mildly preserved products have a short shelf life, of about 5 days at chill. Spoilage is caused by the lactic acid bacteria and quality loss is taken as loss of color. They may contain infectious pathogens, such as salmonella and *L. monocytogenes*, because processing and storage do not reduce numbers. They have their origins in a time before agricultural systems based on intensive husbandry increased the incidence of infectious pathogens, such as salmonellas in meat. Currently, it is assumed that the level of infectious pathogens in the raw materials is still low enough to ensure product safety, without any decontamination. However, it is difficult to regard these products as microbiologically safe in view of the the known incidence of infectious pathogens in pork.

Low a_w Products (Dry-Cured Hams, Bacon, and Nonfermented Dried Sausages)

Traditional dry-cured hams are made from whole primal joints dry salted on the outside; salt is applied to the interior, around any bone present (a typical process for dry-cure ham is shown in Table 18-6). Initial processing and storage is generally below 10° to 12°C,[144] in air for a period of some weeks; higher temperatures (20° to 25°C) are used to complete the

Table 18-5 Typical Process for Teewurst

Manufacturing Stages	*Microbiological Importance*
Select and trim chilled pork pieces	Initial level of pathogen contamination will determine safety of product
Mince	Contaminants from one carcass may contaminate others
Blend with dry ingredients, including 0.15% sodium nitrite and salt (minimum a_w 0.95)	
Ferment, using starter, pH <5.3 within 3 days, 15° to 20°C	To provide small reduction in numbers of infectious pathogens (ie, 1 log) and prevent growth of *Staphylococcus aureus*
Portion and pack	
Chill, to below 7°C	
Store and distribute at 5°C; maximum 5 days	

Table 18-6 Typical Process for Production of Dry-Cured Ham

Manufacturing Stages	*Microbiological Importance*
Select and trim chilled pork legs and shoulders	Initial level of contamination will determine safety of the product
Remove bone	
Pack bone cavity with dry crystalline rock salt (contains sodium nitrate) or dry-curing salts	Low a_w interior of joint to prevent microbial growth
Refold meat, to give shape, and tie; rub the surface with dry crystalline rock salt or curing salt	
Dry, initially at <10°C (until the a_w is <0.96), then at a temperature up to 25°C for about 28 days, to give low a_w (eg, 0.7–0.9) depending on stability required	Initial drying is at low temperature to inhibit growth of pathogens
Wash off excess salt and dry at <30°C	Excessive washing will allow a rise in a_w and favor spoilage; high drying temperature may allow growth of *Staphylococcus aureus*
Net wrap or vacuum pack	Net wrapping is suitable for products with an a_w of approx. 0.7; vacuum packing of higher a_w products will prevent growth of molds
Store and distribute, ambient temperature for products with a_w approx. 0.7; chill temperature for products with higher a_w	Temperature changes can promote condensation in vacuum packed products and thus cause spoilage

drying process, after drying to an a_w of less than 0.96. Poor initial cooling of joints, after slaughter, and slow curing or a_w reduction may allow the growth of contaminants eg, *Arthrobacter*, corynebacteria, *Bacillus*, *Sarcina*, and *Aerococcus*.[30,107] As there is no heating during processing, salting and rapid drying to give a_w below about 0.95 is essential to prevent the growth of pathogens[140,190]; the final a_w of raw ham is 0.7–0.8. Slow rates of preservation (weeks rather than hours) and cool temperatures allow survival of *S. aureus* or *C. perfringens* in these and other dry products.[95] To prevent the outgrowth of clostridial spores, the rate of a_w reduction and chilling and holding temperatures must be linked, so that conditions within the joints remain outside germination and outgrowth limits of spore formers. A predictable level of unbound nitrite during processing and shelf life is essential for safety and to prevent spoilage; for typical input levels, *ca.* 150 ppm, residual levels of 10–50 ppm are common. Some hams are preserved only by the use of sodium chloride (12% to 15% w/v) but the salt used contains sodium nitrate, which is reduced by the microbial flora to nitrite. This combines with the meat pigments to give the typical cured color of raw ham. There is a strong interplay, protective or otherwise, with humectants such as salt and proteins and the spoilage flora. Thus, the survival or growth of *S. aureus* in dried sausages may depend on the growth of lactic acid bacteria during processing.[155] Poor hygiene during manufacture will allow the growth of salt-tolerant microbes, such as micrococci,[158] in equipment, and these may be introduced onto the product during curing or by handling. Poor temperature control when hams are washed to remove excess salt may lead to the growth of *S. aureus* and other organisms.[226] The formation of bacterial slimes on these products during storage is rare and the surface flora is dominated by molds (eg, *Aspergillus*, *Monilia*, *Oidium*, *Fusarium*, *Mucor*, and *Penicillium*),[134] including toxinogenic species.[191] Trichinae may also be a hazard in dry-cured hams, but may be eliminated by storage at 24° to 35°C for periods of time depending on size of the ham.[139]

Bacon may also be manufactured using a dry-curing route. Because of the low a_w, its microflora is dominated by salt-tolerant micrococci, yeasts, and molds, similar to dried hams. On slicing, contaminants from the outer surface may be redistributed on the faces of each slice; shelf life is limited by the extent of growth that leads to unacceptable quality changes.

The key microbiological mechanisms at work are inhibition and selection by reduced water activity, salt and nitrite, and restriction of growth by storage temperature. Because low water activity is a key preservative, surface uptake of water may cause localized spoilage; for example, if there is condensation on a cold surface when product is moved from a chill to an unchilled area. Prolonged frozen storage may cause rancidity in these products.[179]

18.4.4 Cooked, Cured Products

These products include cooked hams, comminuted pork products, and pates with closed-pack storage life under chill of up to 2 months (ambient-stable canned products have a shelf life of over 12 months). The preservation system is complex but is principally the combined effect of heat and curing salts (ie, sodium chloride and nitrite) and storage temperature; the nitrite provides the characteristic color of cured products by forming nitroso-compounds with myo- and hemoglobin. The minimum heating process used depends on the intrinsic preservation system and the intended storage temperature. Pasteurization (60° to 80°C) will eliminate all vegetative cells, but does not kill spores. Hence,[75,76] the combination of salt and nitrite in the product and the storage temperature must prevent their growth. The more severe processes (100° to 110°C) used for shelf-stable canned products cause a limited additional reduction in spore numbers and injure the survivors, limiting their ability to outgrow, especially in the presence of nitrite and salt.[92,166]

The most severe microbiological hazard associated with the ambient-stable products is toxin production by growth of *C. botulinum* from spores surviving the heat process or contaminating the product after processing.[93,96,182] Hauschild and Simonsen[92] considered that the botulism risks were controlled by a combination of the low incidence (*ca.* 1 spore kg^{-1}) of *C. botulinum* in the raw materials and the effects of thermal injury in the presence of salt and nitrite on these spores. In cooked products, nitrite is particularly effective at inhibiting the germination and outgrowth of clostridial spores,[157,182,187,215] an effect that is enhanced by chilled storage.[184,185,186] To be effective in ensuring microbiological safety, there must be sufficient nitrite at the beginning of processing to ensure the presence of free or unbound nitrite during and after cooking (typical input levels of *ca.* 150 ppm), and minimum effective residual levels *ca.* 5–10 ppm are needed to inhibit the germination and outgrowth of *C. botulinum* spores.

Heating (>90°C) is believed to enhance the effectiveness of nitrite in two ways. First, it promotes the direct reaction of nitrite with spore components thought to be involved in outgrowth, and second, it causes the formation of inhibitory compounds by reactions with iron-containing compounds in meat.[174] Because the formation of these so-called Perigo factor compounds is so variable, and because there is doubt whether they play a significant role in the safety of cured meat products, their inclusion as a quantifiable part of the cooked cured meat preservation system is not possible.[175]

The antimicrobial effects of nitrite are enhanced by reduced water activity (<0.98). This can result from the addition of salt or other humectants or the solubilization of low molecular weight meat components.[82] Roberts and Gibson[184–186] carried out a series of experiments examining the antimicrobial effects in pork slurries of heat treatment, pH (5.5–6.3), nitrite, and salt level. They concluded that increasing nitrite level did not affect spoilage, but controlled growth of and toxin production by *C. botulinum* Types A and B up to 15°C. The effect was increased by higher heat treatment, salt level, and the presence of ascorbate and lower storage temperature.[185] The use of other agents, such as phosphates, has been investigated as a means of achieving stability of "mild" products.[141] Gamma-irradiation (3–4 kGy) has also been tested as part of a combination preservation system for cooked cured meat products.[70,72]

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Chill-Stable Products. These are made using mild heating processes with coldest point temperatures of 60° to 85°C. Minimum time/temperature combinations are usually above those required for cooking to ensure that the products are free of infectious pathogens. Because the product units cooked are often large (eg, 12 x 15 x 60 cm), process times of 6 to 8 hours at oven temperatures 67° to 75°C are necessary to allow heat penetration to the center of the product. There is a slow rate of heating because of the small temperature difference between the product target temperature and the oven temperature; this is necessary to prevent the cook-out of gelatin. This means that much of the killing is done at low temperatures of 50° to 60°C, where its kinetics are poorly understood. The vegetative microflora is destroyed, but the majority of the bacterial spores remain viable. However, heat interacts with nitrite to inhibit surviving spores.[166] Because product yield is maximized by the use of the lowest possible heat process, the survival and growth of unusually heat-resistant vegetative cells may occasionally cause spoilage, for example, by the ropy-slime producer *L. viridescens*[76,142] or by streptococci. This type of spoilage may occur when high temperatures (>15° to 20°C) occur during raw material transport, storage, or the curing process and have allowed microbial numbers to exceed 10^6 g^{-1}.[143] To remedy this, heat processes may have to be increased if there is a build-up of heat-resistant species, such as enterococci.[168] Spore outgrowth will be affected by the rate of cooling after heating and rapid cooling to 8°C increases stability.[113] The length of time between 55°C and *ca.* 20°C should be limited to 2 to 4 hours to prevent the growth of mesophiles and activation of spores, and further cooling from

20°C to 5°–10°C should occur within 8 to 12 hours. Slow cooling to temperatures above 20°C may lead to the outgrowth of spores of *C. perfringens*, especially in containers providing anaerobic conditions, such as in clamps or vacuum packs.

When products are removed from the containers used for their cooking, repackaging, slicing, portioning, or resale, the major hazard is recontamination. Only products cooked and stored in the same hermetically sealed container will be shelf stable or stable for prolonged periods at chill temperature (eg, 6 months at *ca.* 3°C), because recontamination is prevented. Various methods have been investigated and used for decontaminating open packs including the use of a hot water dipping, steam,[42] and acid treatment.[167] The fate of any contaminants introduced after cooking will be determined by the intrinsic preservation system[68] and conditions of storage. For instance, in mildly cured vacuum packed product stored at 24°C, numbers of salmonellas increased from 100 to 1 million g^{-1} over 6 days, whereas below 15°C their numbers did not increase.[45] *Staphylococcus aureus* from the hands of food handlers may contaminate products and at temperatures above 10°C may grow and form enterotoxins. Other pathogens such as *L. monocytogenes* may be picked up from equipment or cooling facilities and grow at chilled temperatures[55,202] and cause illness.[150] Many methods have been proposed to protect products from pathogen growth. For example, reduction of the a_w from 0.993 to 0.96, pH reduction from 6.9 to 5.9,[55,201] high CO_2, salt, and nitrite levels and low pH with additional chemical preservatives, such as lactate, sorbate, or nisin,[25,29] have been proposed to prevent growth of *L. monocytogenes* during chilled storage.

The most common post-process contaminants are spoilage types, because they are most numerous in the processing area or on equipment. In sliced, vacuum packaged luncheon meats, storage temperature will determine to a large extent whether spoilage is caused by lactic acid bacteria or by *B. thermosphacta*,[60] the latter being favored by lower temperatures and the presence of nitrite.[169] Both species may be found in slicing areas and the risk of contamination is controlled by process hygiene, which includes use of a forward-flow process layout, the segregation of personnel handling raw and cooked products, and implementation of operating procedures designed to prevent cross- and recontamination after cooking.[36] If there is poor separation of raw and cooked material during retail sale, especially if products are unwrapped, then contamination with infectious pathogens such as salmonella, verocytotoxigenic *E. coli*, or *L. monocytogenes* can occur.[85,104,159] This type of product is often sold sliced, and slicing and vacuum packing within the manufacturing environment, rather than at a retail outlet, offers the best chance of preventing recontamination, because segregation and hygiene procedures are easier to control. The film or web materials used for vacuum or modified atmosphere packaging (MAP) and the equipment used must give high degrees of seal integrity and low permeabilities for CO_2 and oxygen, to contain the gaseous atmosphere,[46] which reduces the growth rate of spoilage microorganisms.[57] Modified atmosphere packaging using carbon dioxide as the atmosphere generally encourages the growth of lactic acid bacteria, such as leuconostocs,[56,58,121] and suppresses the growth of the aerobic Gram-negative bacteria on this type of product. *Yersinia enterocolitica* will survive in chill-distributed frankfurter-type sausages.[6]

Ambient-Stable Products. Where there is a requirement for ambient stability, fractional F_0 or full sterilization processes are normally used. The choice of heat process should be governed by the spore challenge from the raw materials[92] and the concentration of curing salts used.[108] Thus, the higher the initial concentration of spores in the raw materials prior to heat sterilization, or the lower the concentration of curing salts, the higher the heat process that should be used to achieve microbiological stability. Unbound nitrite at the end of the heat treatment is essential for microbiological stability. Ascorbate is used to reduce nitrite losses during processing and storage,[145] but some ingredients, such as MRM, which may have a high iron content,[117] may increase nitrite loss.[47,48] Other ingredients, such as soy protein, may also reduce the effectiveness of nitrite.[114] If the input levels of salt and nitrite are high (nitrite > 150 ppm + salt/water >3.5%), then it is possible to use a fractional F_0 process (typically 0.05–0.86, but up to 1.5), giving limited log reductions of *C. botulinum* (<2.5D). In some countries the risks associated with these low heat preserved products are increased by the consumers' wish for low salt (<3.0% s/w) (and therefore low nitrite) products. Such salt-on-water levels in the presence of low residual nitrite (<5 ppm) are insufficient to inhibit the growth of *C. botulinum* and other clostridia, under ambient storage conditions. To minimize the chances of spoilage and to provide a good margin of safety, higher minimum processes (F_0 *ca.* 1.5–3.0) should be used for low-salt and/or low-fat ambient-stable hams and luncheon meat–type products.

After cooking, products are cooled rapidly to inhibit spore germination and to limit the adverse effects of heat on texture and cook-out of gelatin. Because spores may not have been killed in sufficient numbers by the temperatures and times involved in cooking, rapid cooling is essential to prevent their repair, germination, and growth. This allows them to overcome the stabilizing effects of the preservation system. The length of time for initial cooling from process temperatures (*ca.* 105°C) to about 65°C is usually rapid. If cans are cooled by a water shower in a retort, then exit temperature should be sufficiently high to promote drying, by evaporation (>45°C), but low enough to prevent can distortion by internal overpressure. Holding time between 55°C and *ca.* 20°C should be limited (*ca.* 8 hours) to prevent spore repair. Hygienic handling of cans after heat processing is essential to prevent post-process contamination of the can contents.[36,194] The water used for cooling the containers after heating must be decontaminated to prevent pack recontamination; this is usually done by chlorination (*typically* 0.2–5 ppm free or residual Cl_2). Measures to prevent recontamination include the use of clean, segregated areas, which are free of non–heat-treated food materials and personnel who may have been in contact with such materials. To minimize risks of recontamination, the post-process areas need to be maintained to very high standards of hygiene by the regular cleaning and disinfection of the environment and equipment. These products are usually sold in their primary packaging or vacuum pack bags, although they may be unwrapped after storage for slicing, etc. Once the primary pack has been opened, it should be treated as chill stable. A typical process for the manufacture of canned luncheon meat is shown in Table 18-7.

Table 18-7 Typical Process for the Manufacture of Canned Luncheon Meat

Process Stages	*Microbiological Importance*
Selection of raw materials including meat, fat, MRM, and blood	The bacterial spore load (max level of spores should be 100 g^{-1}) and the ability of the meat mix to bind nitrite
Size reduction and emulsion formation, addition of salt (*ca.* 2.5%), nitrite (150 ppm), ascorbate and stabilizer (eg, disodium diphosphate)	Formation of intrinsic preservation system
Mixing of additional dry ingredients, cereal	
Chilling	To give viscosity required for filling
Filling into cans	Weight control to achieve effective and consistent thermal process
Vacuum closing and can seaming	To prevent contamination of contents
Thermal processing and cooling of cans	Fractional F_0 0.15 (process temperatures *ca.* 105° to 110°C) customarily used; for low-salt products (<3% s/w) an F_0 1.5–3 process should be used and cooling to 20° to 30°C should be complete within 10 to 12 hours. Residual level of nitrite 5–10 ppm
Post retort can handling	Prevent recontamination of wet cans through their seams
Storage	Products are designed for storage <25°C

18.4.5 Commercially Sterile Meat Products

Typical products are canned meats and meat-containing soups sterilized in cans, jars, or pouches. They may also be in-line sterilized and then aseptically packed. These products can support the growth of most pathogens because they do not contain an intrinsic preservation system, thus commercial sterilization is required. The heat processes used are at least equivalent to 3 minutes at 121°C, ($\geq F_0$ 3), the minimum mandatory requirement for safety in many countries, and considered sufficient to cause a 12-D reduction in numbers of *C. botulinum*. Commercially used processes (eg, $F_0 \geq 5$–8) are usually above this minimum to eliminate the more heat-resistant spores of species able to cause spoilage at temperate (<35°C) storage temperatures. Packs of these products may be either conduction or convection heated, which affects the rate of heat penetration. In addition, they may not heat uniformly if they contain mixtures of particles and liquids, therefore solid-liquid ratios should be specified. Other process and product factors can also affect the rate of heat penetration. Major variables include the product temperature at the start of the thermal process, eg, frozen particulates which may be used and the effect of these on the initial temperature of the product at the commencement of the report cycle must be determined.[193] Also the effect of ingredients on product viscosity must be determined. Unpredictably high viscosity can reduce the rate of heat penetration, and hence the heat treatment received for particular process conditions. Compositional factors, such as high oil levels in emulsified products, may reduce the lethality of heat processes by increasing spore heat resistance in a low a_w environment or causing increased tailing of survivor curves.[80] Usually the heat processes used for meat products are not sufficient to ensure that they are completely free of the heat-resistant spores of thermophiles. Severe sterilization processes (F_0 15–35) are required for their elimination; and these treatments are likely to have a marked detrimental effect on product quality. The risks from thermophile spoilage (caused by *Bacillus* or *Clostridium* spores) are accepted as low in meat products, because their occurrence in sufficient numbers to survive standard heat processes is rare, even in flour and spices ingredients.

Microbiologically Important Features of the Manufacturing and Formulation Technology

The most important preservation features are the designated minimum heat process ($\geq F_0 = 3$), and prevention of post-thermal processing recontamination by an assured level of pack integrity, hygienic handling post-thermal processing, and prevention of pack damage. To meet the designated process reliably, it is essential to use containers and sterilization equipment with predictable heat penetration and heating characteristics and to control filling and sealing procedures. Hygienic cooling and prevention of manual handling of processed containers while they are warm and wet is essential to prevent spoilage by recontamination. The equipment used for handling containers must not damage them and must be hygienic. For further information on the thermal processing of food see Chapter 3.

18.4.6 Low and Intermediate a_w Products

These products rely mainly on low water activity for stability during ambient storage (typical shelf life is 12 months). They include those traditionally made without heating (eg, sun- or air-dried meats; $a_w < 0.6$) or those produced industrially, often with mild heating for setting the proteins, followed by air, spray, or freeze drying (typical moisture contents of <5%, a_w <0.1). Meat extracts (typical a_w <0.8) are also produced from bones, etc, by very high heat rendering treatment (>130° to 140°C) and evaporation plus salt addition (>12% to 20% salt) to provide stability. Intermediate moisture meat products may be made using a variety of humectants; examples of such products are described in Chapter 14.

The microflora surviving in mild-heated products (such as air-dried meats) includes Gram-positive, catalase-positive cocci, eg, staphylococci and micrococci.[122,190] Pathogens such as *S. aureus* and salmonella may also survive mild heat (<50°C) drying processes, but may die out during storage.[120] Freeze drying causes little or no reduction in microbial numbers. Meat products produced by high temperature rendering are usually sterile, and any microorganisms present will be factory contaminants, eg, halo-tolerant species such as yeasts and molds. Hence, rehydration during storage or use can lead to products becoming moldy.

To allow air drying within a short time, meats in industrial processes (Table 18-8) are usually broken

Table 18-8 Typical Process for an Industrial Dried Meat Product

Process Stages	*Microbiological Importance*
Selection of meat pieces	Initial concentration of pathogens
Trimming and removal of fat and bones	
Pre-breaking and flaking of meat	To provide small particles (*ca.* 5 mm) capable of rapid a_w equilibration
Preparation of stock from bones (100° to 140°C for 3 to 4 hours)	Stock is usually sterile unless recontaminated during the downstream process
Blending of meat flakes and stock	
Low temperature (50° to 60°C) heat setting of proteins in stock solution	Temperatures only provide minimal pasteurization
Drying by evaporation, cooling, and cutting to give particulates	May lead to recontamination (equipment and environment); effective and uniform drying for initial reduction of a_w is essential
Final air drying of particulates (typical maximum process temperature 75°C)	Low a_w (*ca.* 0.1–0.4) may give increased heat resistance to pathogens
Milling of dry particulates	May lead to recontamination, as complex pieces of equipment are difficult to clean and if wet cleaned, difficult to dry
Filling into moisture-proof bags	Maintenance of low a_w by pack integrity

down into small particles (<5 mm) or in the production of traditional dried meat products such as Biltong into very thin strips having a similar maximum thickness; control of size and hence drying rate is critical to safety. Because of the complexity and scale of the industrial scale plant used, effective control of hygiene and the prevention of cross-contamination is difficult. It is a major decision whether microbiological safety is best protected by the use of wet cleaning and disinfection procedures on plant equipment known to be difficult to clean and dry, or whether dry cleaning methods will give a lower overall risk, because growth is prevented. Where continuous processes are used, "in-process" material often has a substantial range of residence times and moist material may be held up for potentially hazardous periods at growth temperatures. Therefore equipment should be designed and controlled to minimize the build-up of materials and long drying times at temperatures allowing microbial growth.

Microbiologically Important Features of the Manufacturing and Formulation Technology

Dehydration, drying, or addition of humectants (eg, salt, glycerol, and sugars, may be used for intermediate a_w products) are used to reduce the a_w to <0.85 or usually to <0.6. Inhibitory water activities must be achieved sufficiently quickly to prevent microbial growth and toxin formation. Heat used for setting process (Table 18-8) should be sufficient to destroy likely levels of infectious pathogens present in raw materials. After heat processing, cross- and recontamination must be prevented. The design and operation of continuous processes must ensure that these are suitable means of preventing the fastest moving materials within the main product flow receiving less than the time and temperature required for proper drying. Dried meats are hydroscopic, hence their primary packaging must be done as soon as possible after processing. Where containers or packs are incompletely filled, or there are temperature fluctuations during storage, a_w may increase because of condensation and product rehydration, and mold growth may occur within the pack. This may be minimized by reducing the head-space volume and the risks of mold contamination minimized by hygienic filling. If foods are formulated with ingredients of different moisture contents, or packaged with an inadequate moisture barrier, moisture migration or uptake will raise the a_w to levels allowing spoilage.

18.5 CONCLUDING REMARKS

18.5.1 Manufacture and Product Use

The scale of industrial manufacture of meat products increases year after year with the elimination of many low capacity and small-scale local producers. This offers opportunities for more consistent quality and safety standards via improved control, but reduces the availability of many traditional and specialized meat products. The consequences of any undetected loss of process control during the large-scale manufacturing and wide distribution of large batches of product may have far-reaching effects. Because of the scale involved and the increasingly wide geographical distribution of products, remedial actions, such as product recall, are complicated and impose a requirement for a high level of traceability. The use of global manufacturing (meat products for Europe may be made in South America and the Far East) and multistage distribution systems makes retention of batch integrity and control of the distribution chain difficult. Recognition of the need to limit risk to customers and businesses has lead to increased legislative requirements for producers to apply HACCP, to maintain full processing and distribution records,[192,194,210] and to use product coding allowing good traceability of batches.

Historically, public awareness of food safety, in terms of knowledge of pathogens and safe cooking practices, has not been found to affect customer behavior.[149] Generally, processed meat products, particularly those packaged as a meal or meal component, are considered by consumers as safe and ready-to-eat, and that the purpose of cooking is solely preparation for eating. However, where meat products appear obviously uncooked, and there is sufficient publicity and clear labeling with validated cooking instructions, customer attitudes toward the importance of cooking are, according to some recent surveys in the UK, improving. This is despite the fact that the majority of consumers do not read cooking instructions and do not know the correct temperature for the storage of chill-stored foods. There continues to be a need for more consumer education and hygiene awareness information. The manufacturer may apply a process that changes the surface characteristic of a meat product such that it appears cooked, eg, flash frying. Such products are particularly dangerous because, although they may appear cooked, they are essentially a raw meat product.[225]

Opportunities to manufacture more mildly processed, but microbiologically safe, meat products are provided by the improved ability to operate manu-

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facturing processes close to their "limit values." Microprocessor (PLC) controls allied to multipoint measurement in process equipment allows more accurate and reliable process control than is possible with single point measurement and manual controls. Such improvements allow less separation between the operating targets and limits. Reduced heating conditions are also possible where ovens and retorts can work accurately (± 0.5°C) and reproducibly (no loss of performance over 10,000 operating cycles). Preparation equipment (dosing equipment, fillers, mixers, etc) can give products with very consistent and predictable heating characteristics. Many manufacturers and retailers also rely on their ability to limit contamination of decontaminated products by the use of hygienic slicing and packaging areas for chilled ready-to-eat products.[36] Control of the chill chain to low temperatures (0° to 5°C) slows spoilage and limits the growth of most pathogens. Although there may be improved chill chain handling during distribution, there is no evidence that storage temperatures in the home have been reduced to a similar extent. Increased risks are associated with poor temperature control during domestic storage, which may be the majority of the product's shelf life, and the demand for long "use by" periods increases these risks. Modeling studies may be used to simulate the effects of storage or system failures, and HACCP studies are the only approach for ensuring that these and other risks are adequately considered.

18.5.2 The Use of HACCP and Predictive Models

HACCP is increasingly used internationally by meat product producers to predict hazards and to reduce the risks of products causing foodborne illness.[123,144,192] The use of structured hazard analysis to provide a focus for HACCP studies and the use of data from line audits in these studies and their reliable implementation as management and control systems will significantly improve product safety.[15,27,223] At present, assurance of raw material quality and implementation of HACCP studies based on sound data and expert knowledge of microbiological hazards and their control during production and manufacturing processes are the weakest link in the chain of risk reduction. HACCP studies should be based on line audits and must define requirements in the context of actual process capability. Statistical process control techniques are becoming widely used for assessing their value. Unless the validity of a HACCP study and the quality of its implementation are regularly reviewed, newly identified foodborne pathogens (such as *E. coli* O157:H7) or altered or unexpected hazards (such as ingredients like MRM that bind nitrite) may be missed. As better support tools become available to microbiologists, such as predictive models or epidemiological data, the identification of realistic hazards will improve. The necessity for good HACCP studies in capital and labor-intensive processes, such as those used in meat product manufacture, is increased by continuing pressure to reduce manufacturing costs, especially the *indirect* costs associated with the control or assurance of quality and safety, while maintaining or reducing levels of risk to consumers.[23,210]

Simple D and z models have been used for more than 50 years to predict the lethality of heating processes and to answer "what if" questions concerning the impact of changes in time and temperature during heat processing on the chances of survivors (see Chapter 3). However, these models do not take account of the nonlinear death kinetics often observed. Models based on an understanding of the biological mechanisms involved could allow better design of heat processess to control spoilage and ensure safety. Many mathematical models predicting microbial growth[148] are now available (see below) but few on microbial survival. They can be used for reducing development costs and managing risk, because they allow the user to predict the likely performance of preservation systems in meat products, relying on a combination of intrinsic (eg, low a_w, nitrite, preservatives, or acid) and extrinsic (eg, heat, modified atmosphere, or storage temperature) preservation factors without doing numerous challenge studies. Improved accessibility of models for product developers and the incorporation of an "expert" or at least user-friendly front end would improve their rate of usage.

Because of the complexity of the interactions involved, it is often difficult to pinpoint the critical synergies in preservation systems and predict the effect of ingredients in combination or with nutrients, especially under marginal or stress conditions. Models can be used for identifying the likely critical relationships controlling spoilage or safety hazards, by integrating the effects of temperature fluctuations during cooling, distribution, retail display, and home storage.[26,88,124,169] Mathematical models are available for the major spoilage and pathogenic types and for many of the intrinsic factors (eg, pH and a_w) associated with meat products. These include the following: models of the effect of various parameters on the growth of Enterobacteriaceae and clostridia[12]; *Listeria monocytogenes*[25,68,100,101,103] (a survival model[235]); *Escherichia coli* O157:H7[28]; *Clostridium botuli-*

num[188,189]; and *Yersinia enterocolitica*.[218] A prerequisite for accurate predictions is definition of all the critical physical and chemical product variables. Only when this is done can accurate predictions of growth or toxigenisis under the boundary conditions be made, as there are likely to be differences between model predictions (usually fail-safe) and the real situation in food. Significant interactions between microorganisms may reduce the accuracy of model predictions, eg, *Y. enterocolitica* and *L. monocytogenes* are both additionally inhibited by other contaminants in meat products.[103] Model predictions based on the use of laboratory media need to be validated in appropriate food products (see Chapter 16).

18.5.3 Emerging Pathogen Concerns

The emergence of newly identified[51,53,54] or adapted pathogens with enhanced resistance to existing preservation systems and processes is a major concern in an industry trying to move toward milder products and processes (eg, heat or fermentation processes targeted at salmonella may not be equally effective against *E. coli* O157:H7). These verocytotoxigenic *E. coli* provide a more severe food poisoning hazard than salmonella,[34] so that the reliability of processes used in manufacturing, at home, or in food service to ensure its absence has to be increased. In some cases the identification of food poisoning problems by epidemiological studies has prompted examination of the basis of safety rules. This has in some cases shown that the microbiological analytical methods used to assess the effects of a killing step may not have recovered sublethally damaged cells and therefore overestimated process effectiveness; prior exposure to sublethal stress can increase the resistance of *E. coli* and *L. monocytogenes* to heat[4] and acid.[89] In some cases, incorrect data of this kind has been translated into process requirements.

18.4.5 Microbial Destruction Techniques

Many products rely on heating (either pasteurization or sterilization) to kill microorganisms. Hence, heating is a major contributor to safety and stability, but may cause quality loss. This loss often results from the excessive heating required to achieve "coldest point temperatures." Hence, more uniform and accurate methods will be commercialized to minimize the heat input, eg, volumetric heating, using ohmic or microwave technology. The aim is to reduce energy costs and to achieve processes that are nearer to the minimum for specified product core temperatures and hence, lethalities.

Because inactivation is an essential part of preservation, an increasing range of technologies will be used to do this in future. Ultrahigh pressure (1,000–6,000 atm) has been used experimentally to decontaminate pork slurries and meat products; pressures in the range 3,000–6,000 atm for 10 minutes at 25°C kill vegetative cells (*E. coli*), but >6,000 atm is needed to inactivate bacterial spores (see Chapter 13).[127,208] Currently, the application of such methods is limited by variability in product characteristics, the cost, and low throughput of equipment and as the "unconventional" heating methods are currently "out-of-pack," their full exploitation relies on the availability of economically priced aseptic handling and packaging systems for slices and pieces.

There is international interest in the use of irradiation (either from cobalt or electron-beam accelerator sources)[71] to pasteurize or sterilize meat products.[216] However, before irradiation technologies can be used on any scale, there are two major hurdles to be overcome: consumer acceptance and the prevention of unacceptable product quality loss due to free radical reactions.

18.5.5 Stabilization Methods

Technological advances have concentrated on killing. There has been little application of innovative means of stabilization, ie, improvement of intrinsic preservation systems, such as the use of nisin and other natural antimicrobials in meat products.[29,39,153,206] This may be because of consumer and legislative conservatism[176] or doubts concerning their effectiveness. Pressure from consumers, or marketing departments, asks for healthier products with less fat, and therefore by inference, contain more water. This, coupled with a desire for low-salt products, gives lower salt-on-water concentrations and increases the risks of food poisoning as preservation systems become less robust.

There are many products below the generally accepted minimum 3% to 3.5% salt-on-water considered necessary for preventing the growth of psychrotrophic *C. botulinum* at chill temperatures (<12°C), and yet there is no marketplace evidence that they are hazardous. Consumer pressure for the removal or limitation of nitrite and salt continues and is reinforced by labeling legislation.[31,32] This pressure has led to a continuing search for alternatives such as lactate (2% to 2.5%) and increased reliance on chill storage.[100,171] The inhibition of pathogens in meat products relies increasingly on chill temperatures and less on the intrinsic (ie, pH, a_w, and salt) factors that are traditionally used. Improvements in manufacturing hygiene are reducing the important protective contribution of the spoilage flora and may increase the risks of unlimited growth of accidental, pathogenic, contaminants, such as *L. monocytogenes*. A major challenge to the industry is to conserve the low level of risk associated with the presence of a spoilage flora, while inhibiting its activity, so that long shelf lives at chill are achievable.

18.5.6 Selection/Handling of Raw Materials

The number of microbial pathogens and spoilage organisms present in raw materials can have a critical effect both on the microbiological challenge and the stability of a preservation system. There is little or no data on the contribution that the relatively low incidence of pathogens (eg, *C. botulinum* or *S. aureus*) in raw materials makes to product safety. Their assumed low incidence, coupled with improved hygiene and chilled storage, has been proposed by Skovgaard[212] as an alternative to the use of nitrite. However, even according to this author, the use of nitrite remains justified because of the difficulties of controlling hygiene and storage temperature. As raw materials are sourced on a global basis, established processes may be challenged with more, or different, microorganisms with different or increased heat or preservative resistances. Coverage of this is an essential part of hazard analysis and risk assessment.

Over the past 40 to 50 years, there has been an increased, but now reduced, use of natural spices in meat products. This reduction has occurred because of the availability of "extracts" of good sensory quality and an appreciation that the risks of spoilage are increased in mild products (such as luncheon meat), which become microbiologically unstable when challenged with high spore loads. Since 1986, input and residual levels of salt and nitrite in cured products have been reduced (from 300 to 150 ppm), but the geographically wide market for these products has meant that they are now subject to higher storage temperatures than envisaged in the original product design.[92] Therefore, it is not possible to conclude from their good safety record whether current usage is a well-controlled risk or whether there is only absence of evidence of failure.

Packaging materials, such as barrier films or cans, prevent microbiological recontamination and retain preservative atmospheres. Pressures to reduce packaging costs and eliminate "apparently" superfluous packaging material continually erodes the reliability that can be placed on this element of the preservation system. Loss of pack integrity by accidental damage may allow either contamination or a change to the preservation system, allowing spoilage or at worst toxin production. At present, clear guidelines on requirements for packaging functionality do not exist, and packaging should be treated as a key element in HACCP studies.

Increased preparation of processed meat products in food service outlets provides an opportunity for better control of distribution, storage, and preparation hygiene and cooking conditions. However, these tasks are often undertaken by poorly trained staff and within severe cost constraints. This has an adverse effect on product safety because of poor personal hygiene and excessive handling of foods leading to cross-contamination and inadequate temperature control. The common contaminants in shops selling raw and cooked meat are *E. coli* and *Enterococcus faecalis*.[23,221,222] On the positive side, many of the successful multiple retail stores and food service outlets in this sector of the market in Europe and North America have been in the vanguard of pushing high standards of food hygiene and safety, with very thorough employee training, control, and QA systems. Because of the costs of enforcement, and the recognition that quality and safety cannot be ensured by traditional inspection and product testing regimes, there are global moves toward industry self-regulation with the greater emphasis on companies basing product safety on process design and the control of processing by the application of HACCP and quality management systems (see Chapter 58).

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19 Fermented Meats

Friedrich-Karl Lücke

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19.1 INTRODUCTION

Raw salted meat products may be subdivided into three categories. In the manufacture of the first of these, whole muscle products like bacon or comminuted products such as Chinese-style raw sausage (mainly preserved by drying at temperatures around 50°C[102]), there is little, if any, enzymatic activity involved. In the second, various whole pieces of salted or cured meat undergo a ripening process involving tissue enzymes (ie, longer than just necessary for development of curing color and even distribution of salt), but the contribution of microorganisms to their sensory properties is small or absent. Such whole muscle products include aged "raw hams" as common in Continental Europe, as well as some other cuts of pork (eg, Italian "coppa") or from beef (eg, "pasterma," "Bündnerfleisch," or "bresaola"). The third category of fermented meats depends, by definition, on microbial activity. This applies, with few exceptions, to fermented sausages only. Hence, this chapter will focus on the microbiology of sausage fermentation and will only briefly mention possible roles of microorganisms in the manufacture of other meats. The other two categories of raw salted and/or cured meats are discussed in Chapter 18 of this volume.

19.2 TRADITIONAL TYPES OF FERMENTED MEATS AND THEIR MANUFACTURE

Because traditional ripening procedures require low to moderate temperatures, fermented meats are most widespread in regions with temperate climate.

Fermented sausages probably originated in the Mediterranean regions, whereas raw, salted, unground meats were more common in regions with colder, maritime climates. The few varieties of fermented meats indigenous to countries with hot climates include only products that can be dried rapidly and/or are eaten after cooking, eg, *nham*, a fermented sausage common in Thailand. A flow diagram of some typical processes used for the manufacture of fermented sausages is shown in Section 19.6.4 (Figure 19-2) and a list of the types of fermented sausages in Table 19-1. A more detailed list of the types of fermented meats is provided in several references.[18,102,196]

Fermented sausages are made from comminuted meat and fat, salt, curing agents, sugar, and spices. The mixture is filled into casings and subjected to a ripening process. Ripening comprises a *fermentation phase,* characterized by microbial activity, and, in most cases, an *ageing phase,* where the products are dried and develop typical aroma and flavor by complex reactions involving tissue or microbial enzymes or abiotic transformations. *Curing* means a treatment of the meat with curing agents (nitrite, mostly in combination with ascorbate, or nitrate), which brings about characteristic sensory changes (formation of curing color and curing aroma and flavor). These definitions will be used throughout this review.

About 750,000 tons of fermented sausages are produced annually within the European Union (EU).[55,113] Germany has the highest *per capita* consumption (about 4.5 kg per year), while in the United Kingdom (UK) and Ireland, consumption figures are very low, and there is little domestic production. In the United States (US), the annual *per capita* consumption is about 0.6 kg.[1]

There are various ways of classifying fermented sausages; one such classification is shown in Table 19-1. The most important criterion used is the water activity (a_w) of the product because this determines not only its market value but also its microbial stability. Traditional raw sausages with an a_w below 0.90 are, in this chapter, termed *dry sausages.* The a_w of *semi-dry* sausages is between 0.90 and 0.95, and they should be stored below 15°C. Spreadable sausages (a_w 0.94–0.96) lose little if any moisture during ripening, and their safety and shelf life are mainly ensured by pH. Another important factor that affects both the microbiological and the sensory properties of the sausage is the treatment of the sausage surface. Sausages that are not smoked develop a surface flora (molds, yeasts, Gram-positive cocci), which may be either washed off (as in some German-type air-dried sausages) or left on to add to the sensory properties of the product. Such mold-ripened sausages predominate in Italy, France, Spain, Switzerland, and Hungary, whereas in other countries including the US, most fermented sausages are smoked.

Sausages belonging to the different categories (Table 19-1) also differ with respect to their formulations and fermentation conditions. A general rule is

Table 19-1 Classification of Fermented Sausages

Category	*Ripening Times*	*Final Water Activity*	*Application of Smoke*	*Examples*
Dry, mold fermented	>4 weeks	<0.90	no	traditional Italian salami French "saucisson sec"
Dry, mold fermented	>4 weeks	<0.90	yes (during fermentation)	traditional Hungarian salami
Dry, no mold growth	>4 weeks	<0.90	yes or no	German "Dauerwurst"
Semi-dry, mold fermented	<4 weeks	0.90–0.95	no	various French and Spanish fermented sausages
Semi-dry, no mold growth	<4 weeks (usually 10–20 days)	0.90–0.95	yes (with exceptions)	most fermented sausages in Germany, The Netherlands, Scandinavia, US, etc
Undried, spreadable	<2 weeks	0.94–0.96	yes or no	German "Streichmettwurst"; Spanish "sobrasada"

Source: Reprinted from F.K. Lücke, Fermented Sausages, in *Microbiology of Fermented Foods,* 2nd ed., Vol. 2, B.J.B. Wood, ed., pp. 441–483, © 1998, Aspen Publishers, Inc.

that fully dry sausages with a long shelf life are fermented more slowly (ie, at lower temperatures) than the others, and care is taken to avoid excessive acid formation. Likewise, products intended to develop a surface flora are fermented more slowly than sausages to be smoked. The microbiological basis for this will be discussed in Section 19.4.1. Other parameters used to classify fermented sausages include the following:

1. the casing diameter
2. the degree of comminution of the ingredients
3. the animal species used for obtaining the raw material
4. the fat content and type of fatty tissue used
5. the use of special spices and seasonings
6. the types and proportions of other nonmeat ingredients.

19.2.1 Choice and Preparation of Raw Material

Fresh, raw, sausage mixture contains 50% to 70% lean meat, that is, mammalian (sometimes poultry) skeletal muscle tissue. Meat availability (and price), religious traditions, and eating habits determine the animal species. To avoid problems early in fermentation, meat should have a normal pH (5.5–5.8) and a low level of undesired microorganisms, eg, Enterobacteriaceae. For optimal color and consistency of the sausages, butchers prefer meat from old animals such as sows and avoid use of PSE (pale, soft, exudative) pork. The fatty tissue used should consist of fat with a high melting point. For dry sausages with a long shelf life, not more than 12% of the total fatty acid should be polyunsaturated[180]; otherwise, color and flavor defects and rancidity will develop early. Firm back fat from pigs fed a diet with limited levels of polyunsaturated fatty acids is most frequently used. In countries where religious tradition prohibits the consumption of pork, beef tallow or certain other fatty tissues from ruminants are used instead, eg, for Turkish *soudjouk*.[63]

The raw material is cut into appropriate pieces, which are subsequently chilled or frozen. By rapid freezing after slaughter and by avoiding long-term frozen storage, the peroxide content of fatty tissue is kept to a minimum.

19.2.2 Comminution

The raw material may be comminuted in cutters or grinders; the latter brings about a "shorter" (more crumbly) consistency required for various traditional products. It is important to keep the temperature close to the freezing point; otherwise, the efficiency of cutting will be reduced and fats may smear. It is common practice to add part of the raw material in the frozen state.

19.2.3 Additives

Addition of 2.5% to 3% (w/w) salt (sodium chloride) is most common. In conjunction with a moisture content of the mix of about 50% (as in common formulations), this will give an initial a_w of 0.955–0.965. Apart from its effect on the microflora and flavor, salt interacts with the myofibrillar structure and solubilizes proteins, which form a sticky film around meat particles. Nitrite is preferred as curing agent, for undried and semi-dry sausages with maximum input levels of about 150 mg $NaNO_2$ kg^{-1}. In many countries, nitrite may only be applied as a mixture containing about 0.5% sodium nitrite in NaCl. This effectively prevents overdosing of nitrite because such products would taste too salty. Sodium ascorbate (usually 300–500 mg kg^{-1}) is frequently added, together with nitrite, to accelerate the development of curing color and flavor. For dry sausages, manufacturers use nitrate (up to 300 mg KNO_3 kg^{-1}) or reduced levels of nitrite. Some fermented sausages are produced without any curing agents. This is possible if particle size is coarse, sausage diameter is small, and fermentation temperature is low.[192]

Carbohydrates are added to the mix to provide an energy source for lactic acid bacteria, but large amounts of milk powder or starchy products are sometimes used to modify flavor or simply used as a filler; the amount of fermentable carbohydrate needed depends on the initial pH and on the target pH after fermentation. Addition of 0.3% of glucose or another rapidly fermentable mono- or disaccharide will enable lactic acid bacteria to lower the pH by at least 0.5 units, which is sufficient for many products if meat of normal pH (≤ 5.8) is used.

Up to 0.5% glucono-δ-lactone (GdL) is sometimes added to the sausage mixture. It acts as an acidulant because it is hydrolyzed to gluconic acid within a few hours. It is fermented to lactic and acetic acid by various lactic acid bacteria. This may improve microbial safety but at the same time strongly interferes with other reactions required for development and maintenance of desired sensory properties (see below). Hence, GdL is used mainly in the manufacture of "fresh," undried, or semi-dry fermented sausages. The use of starter cultures for fermented sausages is discussed in Section 19.5 of this chapter.

Ground pepper is usually present in all types of fermented sausages at a concentration of 0.2% to 0.3%. Other spices commonly used include paprika, garlic, mace, pimento, and cardamon. Generally, air-dried sausages are more heavily spiced than smoked sausages. Apart from their effects on flavor, spices may also affect the microflora and the oxidative changes during ripening (see below).

19.2.4 Filling

Before filling into casings, oxygen should be excluded from the mixture (eg, by vacuum-filling devices), and the temperature of the mix should not exceed 2°C. Natural casings (made from intestines from slaughter animals) as well as casings made from modified collagen and/or cellulose are most frequently used. They must allow evaporation of water from the sausages and penetration of smoke, and they must follow the shrinkage of the sausage during drying. For undried products, synthetic casings are also used.

19.2.5 Fermentation

Most sausages are fermented at temperatures between 20° and 25°C for 2 to 4 days, although some American-style semi-dry sausages are fermented at temperatures up to 41°C.[9] The higher the temperature, the more rapid the fermentation, but the higher the risk of growth of mesophilic pathogens if acid formation is delayed or inhibited (see Sections 19.4.1 and 19.6.1). Moreover, a high rate and extent of acid formation interferes with the flavor and stability of the product during long-term storage. Hence, lower fermentation temperatures, in conjunction with low levels of fermentable carbohydrates, are applied in the manufacture of dry sausages with long shelf life and for mold-ripened sausages. Hungarian "winter salami"[80] or certain regional specialties in Germany are even fermented at temperatures as low as 8° to 12°C.

To avoid moisture condensation on the sausage surface, the sausages are first equilibrated to the ripening temperature at low relative humidity (RH) before entering the ripening chamber. During fermentation, the RH in the ripening chamber is maintained 5 to 10 units lower than the RH (water activity × 100) within the sausages (ie, about 85% to 90%), and the air velocity controlled at about 0.4 m/s.[178]

In the US, some sausages are heated at the end of the fermentation: regulations require heating to a core temperature of 58.3°C (137°F) if the presence of *Trichinella* cannot be excluded.[9]

19.2.6 Surface Treatment

If a "bloom" on the sausage surface is desired, sausages are dipped in suspension of appropriate starter molds (and/or yeasts) prior to fermentation. If the development of molds and yeasts on the sausage surface during drying or storage is to be prevented, the sausages are smoked, usually at the end of the fermentation period. Some regional specialties in Germany are *air dried,* ie, surface growth is prevented by control of relative humidity and repeated washings. In some countries, natamycin or sorbate dips are used to prevent the surface growth of yeasts and molds.

19.2.7 Ageing/Drying

Sausages are usually aged at 12° to 15°C. The climate in the ripening chamber should ensure a slow but steady drying of the sausages and prevent excessive mold growth on the surface while avoiding uneven drying of the product and the formation of a dry, hard outer layer on the sausages. Hence, the RH in the chamber is gradually lowered to about 75% to 80%, and air velocity is adjusted to about 0.1 m/s. Most fermented sausages are ripened in controlled environment chambers throughout the year. However, many small manufacturers do not have this rather expensive equipment available. Thus, they have to restrict production to suitable seasons or to undried products, or use various means to adjust the climate of the ripening room.

Most sliceable, semi-dry, fermented sausages (about 80% of the products on the German market) are dried to moisture contents of about 40%, which corresponds to a weight loss of about 18% and an a_w value of about 0.93. Dry sausages have 35% moisture or less, corresponding to a weight loss of 25% or more, and an a_w of < 0.90. Obviously, more water must be removed if the formulation includes less fat and more lean meat than normal.

19.3 MICROORGANISMS INVOLVED IN MEAT FERMENTATIONS, AND FACTORS AFFECTING THEM

19.3.1 Lactic Acid Bacteria in Sausage Fermentation

Unless the mixture is inoculated with large amounts of other lactic acid bacteria, lactic acid formation during sausage fermentation is due to the activity of "facultatively heterofermentative" *Lac-*

tobacillus spp., formerly designated as "streptobacteria." These bacteria ferment hexoses to lactate, and pentoses (many strains also gluconate) to lactate and acetate, and grow at 15°C. Early studies showed that the dominating strains could not be identified as validly described species and were therefore designated as atypical lactobacilli.[110] Compared with typical streptobacteria, these atypical lactobacilli (1) are capable of growth at lower temperature (most are able to grow at 4°C), (2) have a somewhat lower pH tolerance (no growth at pH 3.9), and (3) have a higher tendency toward pleomorphism (growth as coccoid cells on low-sugar media).[146,149] A taxonomic study by Kagermeier[86] assigned the atypical lactobacilli to *Lactobacillus sakei* and *Lactobacillus curvatus*. This was later confirmed using more advanced methods.[91] Later, *L. sakei* and *L. curvatus* were also found to dominate the lactic flora in various traditional Spanish,[77,159] Italian,[27] and Greek sausages.[157] Only in some sausage samples from countries with warm climates were mesophilic lactobacilli such as *Lactobacillus plantarum*[86] or *Lactobacillus alimentarius* present in large numbers; the latter species predominate in Turkish *soudjouk*.[65] The data indicate that the reservoirs of psychrotrophic lactobacilli are cold rooms or, even more likely, cutting rooms that are kept at 12°C or below (in accordance with meat hygiene regulations within the European Union), and in which the meat is easily contaminated from reservoirs of psychrotrophic bacteria. It is noteworthy that the species of lactic acid bacteria involved in sausage fermentation are also involved in the spoilage of vacuum packed meat and pasteurized meat products. This also indicates a common reservoir of the strains, namely, the cutting room.

Streptococcus, *Enterococcus*, *Lactococcus*, and *Pediococcus* species cannot compete well with the psychrotrophic lactobacilli at fermentation temperatures commonly employed in Europe and, therefore, comprise only a minor part of the population.[86,146] However, some pediococci have proved to be good starters for various types of sausages (see Section 19.5). Strains producing CO_2 from glucose anaerobi-

Table 19-2 Some Important Properties of Lactic Acid Bacteria Involved in Sausage Fermentations[68,70,91,190]

Property	*L. curvatus*	*L. sake*	*L. plantarum*, *L. pentosus*[b]	*P. pentosaceus*	*P. acidilactici*
Cell shape	rods	rods	rods	cocci in tetrads	cocci in tetrads
Growth at a_w 0.93	–(+)[a]	+(–)[a]	+(–)	V[a]	+
Growth at 4°C	+	+	–	–	–
Growth at 50°	–	–	–	–	+
Peptidoglycan type	Lys-D-Asp	Lys-D-Asp	meso-DAP	Lys-D-Asp	Lys-D-Asp
Lactic acid enantiomer	DL(L)[c]	DL	DL	DL	DL
Nitrate reductase	–	–	V	–	–
Nitrite reductase					
+ heme	–	–	V	V	–
– heme	–	V	V	–	–
Catalase					
+ heme	–	+	V	–	+
– heme	–	V	V	+	–
Ammonia from arginine	–(+)	+	–	+	+
Fermentation[d] of					
maltose	V	V	+(–)	+	–
sucrose	+(–)	+	+(–)	V	V
lactose	V	V	+(–)	V	V
melibiose	–(+)	+	+(–)	V	V
mannitol	–	–	+	–	–
gluconate	–(+)	+	+(–)	V	–
Minimal pH in MRS broth	*ca.* 4.0	*ca.* 4.0	*ca.* 3.7	*ca.* 3.8	*ca.* 3.8

[a] +(–), positive with exceptions; –(+), negative with exceptions; V, variable (strain dependent).

[b] *L. pentosus* is similar to *L. plantarum* but capable of fermenting D-xylose.

[c] Strains forming L-lactate only have been formerly classified as *L. bavaricus*.

[d] All strains ferment glucose and ribose.

cally (*Leuconostoc, Weissella,* and heterofermentative lactobacilli) made up less than 10% of the population. They are undesirable in fermented sausages because they form gas from sugars, and some strains produce slime from sucrose.

Some properties of the species of lactic acid bacteria predominant in fermented sausages and/or available as starters for these products are shown in Table 19-2. All species listed ferment glucose and ribose and produce DL-lactate, except some *L. sakei* strains formerly named *Lactobacillus bavaricus. L. plantarum* possesses both D- and L-lactate dehydrogenases, while *L. sakei* and *L. curvatus* form L-lactate dehydrogenase and racemase.[87] The sugar fermentation pattern and its genetic basis is not very stable in meat lactobacilli, and there is much variation within one species.[45,91,169] Hence, it is important to use the right combination of starter and fermentable sugar.

In the range of relevance to meats, growth of the species listed in Table 19-2 is not affected by pH. Likewise, oxygen is not inhibitory to growth, but may influence the balance of fermentation products by enabling the organisms to oxidize pyruvate to acetate and CO_2. Also, strains that possess flavin-dependent oxidases and little or no peroxide destroying activity accumulate hydrogen peroxide which may cause color and flavor defects (see Section 19.7). Sodium chloride and sodium nitrite, if present at levels equivalent to those in sausage mixtures, lower the rate of acid formation only slightly. The minimum a_w for growth depends somewhat on the strain. In culture media, some strains are only inhibited in the presence of about 12% sodium chloride (approximate a_w 0.91), and acid production in fermented sausages also ceases once the a_w is below 0.91.[98,108] The minimum temperature permitting growth varies between the species; the pediococci as well as *L. plantarum* and *L. pentosus* do not grow at 4°C. This is the main reason why these species are rarely encountered in sausages fermented without starter cultures.

19.3.2 Catalase-positive Cocci

As will be explained later, nonpathogenic, catalase-positive cocci play an important role in forming and stabilizing the desired sensory properties of fermented sausages. Most strains isolated from sausages produced in France,[39] Germany,[54,150] Italy,[27] Spain,[168] and Greece[158] were found to be *Staphylococcus* species. Differentiation into species revealed that *S. xylosus* and *S. saprophyticus* are widespread both in German and French dry sausage as well as in sausages on the British market[39,54,167]; *S. xylosus* dominates in most products manufactured without starter, such as Italian salami.[27] In samples from Germany, *S. carnosus* (originally identified as *S. simulans*) and *Micrococcus varians* are also found frequently.[54] These strains may originate from the starter cultures in use in Germany in the late 1970s.

Staphylococci and micrococci (formerly included in one family, *Micrococcaceae,* but now shown to be only distantly related phylogenetically) have in common that they grow at low water activity (some strains down to a_w 0.86 under otherwise optimal conditions), but are inhibited by low pH and low temperature. Staphylococci are less dependent on oxygen than are micrococci, and this is the main reason why they are found in higher numbers in fermented sausages than the latter. However, growth of all catalase-positive cocci in fermented sausages is restricted to regions close to the surface. High numbers are reached if the products are fermented slowly, not smoked, and if the surface pH rises because of mold growth. In rapidly fermented, smoked sausages, there is hardly any growth of these bacteria.

19.3.3 Yeasts and Molds

The mold flora developing on fermented meats consists of *Penicillium* species, sometimes accompanied by *Scopulariopsis* spp.[84] *Penicillium verrucosum, Penicillium chrysogenum, Penicillium frequentans, Penicillium nalgiovense,* and *Penicillium variabile* are most frequently isolated.[79,105] Aspergilli are rarely found because of their higher temperature optimum. Of the yeasts, *Debaryomyces hansenii* and its imperfect form, *Candida famata,* are best adapted to this environment characterized by low availability of sugars and high levels of salt. Growth of yeasts usually precedes the growth of molds.[5]

19.4 MICROBIOLOGICAL, CHEMICAL, AND PHYSICAL CHANGES DURING MEAT FERMENTATIONS

19.4.1 Sausage Fermentation and Ageing

Day 1: Reactions Involving Oxygen, Nitrite, and/or Gram-Negative Bacteria

After comminution of the meat and filling into casings, oxygen in the sausage mixture is consumed by respiratory enzymes. These enzymes are inhibited by nitrite, which accordingly delays this process. Without nitrite, the oxygen partial pressure approaches zero after about 6 hours whereas up to 24 hours are required after addition of *ca.* 140 mg sodium nitrite kg^{-1}.[152]

If no nitrite is added, the red color of fresh meat is only gradually lost; low partial pressure of oxygen as well as salt accelerate the oxidation of myoglobin to brownish metmyoglobin. The formation of curing color is outlined in Figure 19-1. If sausages have been prepared with added nitrite, they turn brownish rapidly because the oxygenated (red) myoglobin reacts with nitrous acid to give brown metmyoglobin and nitrate. Metmyoglobin then reacts with nitrous acid and/or nitric oxide (from acid-catalyzed disproportionation of nitrous acid) and indigenous and/or exogenous reductants (eg, ascorbate) to give red nitric oxide myoglobin (NOMb), the NO group occupying the sixth ligand position of the heme. The rate of NOMb formation increases with falling pH and is therefore accelerated by the activity of the lactic acid bacteria in fermented sausage. Hence, the formation of cured meat color is normally not complete within the first day.

Aerobic Gram-negative rods (in particular, the psychrotrophs *Pseudomonas fragi, Pseudomonas fluorescens,* and *Pseudomonas lundensis)* make up the majority of the microbial population on aerobically chill-stored fresh meat.[95] Psychrotrophic Enterobacteriaceae are also present while Gram-positive organisms, including lactic acid bacteria, usually occur only in small numbers except if trimmings from meat (particularly beef) are used that have been stored in vacuum packs. Growth of pseudomonads and other aerobic Gram-negative bacteria is readily inhibited at a_w values of about 0.96 (which is the normal a_w of the sausage mix), by lack of oxygen, and by nitrite. Likewise, the degradation of amino acids that would cause the typical off-odors of spoiled meat is largely suppressed. The competitiveness of the Enterobacteriaceae is reduced but not abolished at low oxygen tension, low pH, and in the presence of salt. Their activity within the first 24 hours after filling is largely inhibited by added nitrite. Although this may be desirable in terms of microbiological safety, there is some evidence[192] that nitrite also suppresses the formation of aroma precursors by Gram-negative bacteria early in fermentation. This, along with differences in oxygen content, may be the reason why, empirically, many sausage makers use nitrate or reduced input levels of nitrite for long-ripened dry sausages.

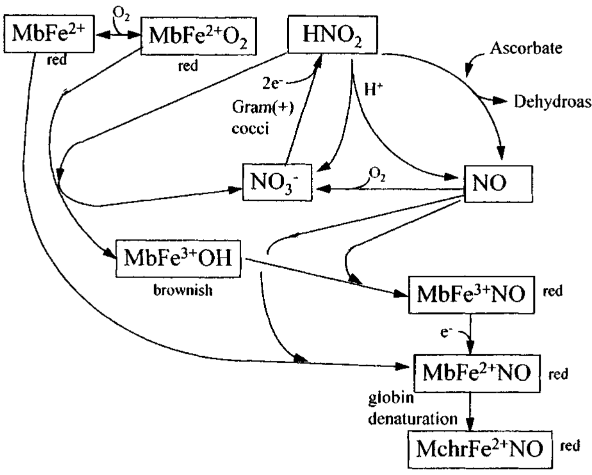

Figure 19-1 Schematic Representation of the Reactions Leading to Cured Meat Color Formation during Sausage Fermentation. $MbFe^{2+}$, myoglobin; $MbFe^{2+}O_2$, oxygenated myoglobin; $MbFe^{3+}OH$, metmyoglobin; $MbFe^{3+}NO$, nitric oxide metmyoglobin; $MbFe^{2+}NO$, nitric oxide myoglobin; $MchrFe^{2+}NO$, nitric oxide myochromogen.

The Fermentation Period Sensu Stricto

The fermentation period *sensu stricto* is characterized by the acid production by lactic acid bacteria, and by completion of cured color formation. The time course of acid formation (apparent lag phase, maximum rate, final pH) is determined by intrinsic (formulation) and extrinsic factors (mainly temperature) in the following ways:

1. A high initial pH of the mixture will have little if any effect on lag and rate but lead to higher final pH values. However, it favors growth of acid-sensitive undesired organisms and may make the process unsafe (see above).
2. Addition of high levels of well-adapted lactic acid bacteria will shorten the apparent lag phase but not necessarily increase rate and extent of acid formation.
3. At low initial a_w (< 0.955), acid formation will be delayed, and rate and amount reduced.
4. At input levels of about 150 mg kg^{-1}, sodium nitrite may somewhat decrease the rate of acid formation[141]; however, its effect on Gram-negative bacteria and listeriae is much stronger than its effect on the lactic acid bacteria.
5. The choice of added sugar affects the length of the lag phase and the rate of acid formation[92,142]; glucose supports fastest and most complete acidification. "Slow" sugars (such as lactose) also tend to give higher final pH values because progressive drying of the product in later stages of the fermentation interferes with acid formation.[108]
6. With increasing levels of rapidly fermentable sugars, more acid will be formed and lower pH values attained.

7. Addition of natural spices may increase the rate of acid formation.[128,187] This is due to their manganese content[195] and the fact that some essential enzymes of lactic acid bacteria including a key enzyme of glycolysis, pyruvate kinase, require manganese.[144]
8. At high fermentation temperatures, the lag before acid production commences tends to be shortened and the rate increased. However, high temperatures may favor mesophilic pathogens and thus, like high initial pH, jeopardize product safety unless appropriate combinations of sugars and starters are used.

If acid production starts too late or proceeds too slowly, pathogens like salmonellae and *Staphylococcus aureus* may grow (see Section 19.6). On the other hand, the activity of the acid-sensitive nonpathogenic staphylococci and micrococci is important, particularly in the manufacture of dry sausage with a long shelf life. To restrict acid formation, such products are made with less sugars and fermented at lower temperatures (see above). Moreover, excessive amounts of acid are undesirable in many fermented sausages and are one of the important causes of product spoilage (see Section 19.7).

The main product of carbohydrate fermentation in ripening sausages is DL-lactic acid, with only small variations between the two enantiomers.[86,108] Most lactic acid bacteria, particularly the "facultatively heterofermentative" lactobacilli predominating in sausage fermentation, also form some acetic acid from ribose or, if alternative electron acceptors (such as oxygen) are available, from pyruvate and/or lactate.[87] After fermentation, average molar ratios of lactic acid to acetic acid of about 7 were found both in smoked[38,40] and unsmoked salami.[61] This is similar to the ratio found in liquid cultures of meat lactobacilli.[135,147] If they excluded oxygen from the sausages by vacuum filling, Deketelaere et al.[38] could recover 88% to 103% of carbohydrate fermented as lactic and acetic acid. Incomplete recovery of carbon indicated loss of carbon dioxide and other volatile compounds from the sausages, which is particularly great in products of small diameter.[41] Small amounts of acetoin, diacetyl, and/or 2,3-butanediol may also be formed by lactic acid bacteria both in culture media and in sausages, and appear to contribute to the "cheesy" component of the sausage aroma.[12,35]

Most semi-dry sausages in Belgium and Germany have pH values of 4.8–5.0 after fermentation, which corresponds to a concentration of approximately 25 g lactic acid kg^{-1} dry weight.[38,108] Some semi-dry fermented sausages produced in the US are even more acidic, whereas in the manufacture of undried sausages the extent of pH decrease is limited in order to keep them spreadable. During the traditional manufacture of Italian and Hungarian salami, the pH remains above 5.3 throughout ripening.[80]

Lowering the pH of the sausages to 5.3 or below inhibits bacteria other than lactobacilli and pediococci, and provides the "sour" component of the sausage aroma. In addition, this pH is close to the isoelectric point of many muscle proteins, and water-binding capacity becomes minimal. Accordingly, the firmness of the sausages rapidly increases, and moisture may be removed easily, but on the other hand it becomes more difficult to maintain the "spreadable" consistency of undried sausages. Lastly, the disproportionation of nitrite and, correspondingly, the formation of cured color is accelerated at low pH.

To be effective as a curing agent, nitrate added as such or formed from nitrite by abiotic reactions (see Section 19.4.1), must be reduced to nitrite by microorganisms. Some strains of *L. plantarum* and *L. pentosus* reduce nitrate *in vitro*, but only when cultivated in media with high pH and little sugar[31,86]; rates are insufficient for proper color and flavor development in meats,[69] and the acid-sensitive catalase-positive cocci are the main agents of nitrate reduction. Therefore, the pH should be above 5.4 until sufficient nitrate is reduced.[130]

Sufficient activity of catalase-positive cocci is not only required for nitrate reduction, but also to form and to stabilize aroma and flavor in the manufacture of sausages with a long shelf life. It is common experience that these sausages should be fermented slowly, and that GdL is unsuitable as an acidulant: this compound is fermented to lactic and acetic acids, and catalase-positive cocci are severely inhibited. Accordingly, GdL sausages are very prone to rancidity and color defects and have only a short shelf life.

Whether added as such or formed by microbial nitrate reduction, nitrite is reduced further by many microorganisms present in the sausage, including the nitrate-reducing microflora as well as several strains of lactic acid bacteria.[25,31,193] This reaction is important to eliminate any nitrite not used for curing reactions. It is sometimes claimed that microbial nitrite reduction is necessary for NOMb formation. However, nitric oxide is not an intermediate of nitrite reduction to ammonia by heme-containing nitrite reductases present in many bacteria including *Staphylococcus carnosus*,[71] which is frequently used as starter culture for fermented sausages. Moreover, sausages fermented with pediococci alone (eg, US-type summer sausages) develop good cured color even though the pediococci do not have nitrite reductases that form nitric oxide.[69] The conflicting results of Garcia et al.,[57] who did not observe curing color formation in aseptically prepared sausage with 100 mg $NaNO_2$ added per kg unless a *Micrococcus* strain had been added, could be due to a massive transformation of added nitrite into nitrate under their processing conditions.

Smoking

Smoke contains volatile antimicrobial compounds such as short-chain fatty acids and aldehydes, and after smoking growth of microorganisms on the sausage surface is inhibited for some time. The products are smoked after the fermentation period although some smoke may be applied earlier if care is taken not to inhibit the fermentation. Phenolic compounds in smoke also act as antioxidants and convey the typical aroma and flavor of smoked products. In the US, fermented sausages made from noncertified pork are usually hot smoked, ie, heated to 58.3°C internal temperature during smoking in order to inactivate trichinellae.

Ageing

Most fermented sausages undergo an ageing procedure characterized by drying (see Section 19.2) and formation of desirable aroma and flavor. Cured color is stabilized because the protein moiety of NOMb is partially denatured and the resulting nitric oxide myochromogen binds nitric oxide more tightly. In unsmoked sausages, a desired "surface bloom" of molds, usually associated with yeasts, develops; otherwise, there is little growth of microorganisms during sausage ageing.

Prior to ageing, the aroma and flavor of fermented sausages are dominated by metabolites from carbohydrates: the products are more or less "sour" and "cheesy," and, if acid formation is restricted, they retain more of the aroma and flavor of fresh meat. At a given pH, a high proportion of acetic acid gives the product a less "pure" and more "sour" flavor.

Most compounds affecting aroma and flavor of an aged sausage originate from lipid and protein degradation. The longer the ripening time, and the higher the activity of microorganisms other than lactic acid bacteria during and after the fermentation period, the higher the levels of volatile compounds with low sensory thresholds that are found.[99,100] Abiotic reactions, tissue enzymes, and microbial enzymes contribute to their formation. A review on the flavor chemistry of fermented sausages has been published recently.[35]

Lipids are precursors of many unbranched aldehydes, 2-alkanones, and short-chain unbranched fatty acids. The first reaction step toward these compounds is lipid peroxidation. This reaction is catalyzed by non-heme iron and partially inhibited by nitrous acid, which binds to this "pro-oxidant." This results in lower levels of volatile carbonyls and a shift in their composition (cured aroma).[64] Attempts to replace nitrite by other antioxidants have had little success[116,191] but certain spices have antioxidative constituents.[67]

The sausage microflora, in particular staphylococci and micrococci, may further modify the carbonyl compounds generated by lipid autoxidation[171] and form compounds, which add to the desired aroma of the sausages. For example, Stahnke[175] reported a positive correlation between the levels of 2-alkanones, salami odor, and numbers of *Staphylococcus xylosus.*

Lipids are also hydrolyzed to free long-chain fatty acids during sausage ripening. These fatty acids have little effect on aroma and, at higher levels, detrimental effects on flavor. However, like lipids, unsaturated fatty acids are further degraded to carbonyls and short-chain fatty acids with peroxides as intermediates.[21] There is some controversy in the literature as to the relative role of tissue and microbial enzymes in lipolysis. Very few strains of lactic acid bacteria from meats[3] but many catalase-positive cocci[29,137] as well as many yeasts and molds from fermented sausages[26,103,104] were found to hydrolyze pork fat (lard) *in vitro.* Cantoni et al.[19] suggested that catalase-positive cocci are the main lipolytic agents during sausage fermentation. The specificity of lipolysis observed by Demeyer et al.[42] supported this finding. Stahnke[174] found elevated amounts of long-chain fatty acids in sausages fermented with a lipolytic *Staphylococcus xylosus* but this had no impact on flavor. On the other hand, lipolysis begins very early in fermentation,[44] and appears to occur even in the absence of catalase-positive cocci or other bacteria.[57] This indicates that tissue lipases rather than microbial lipases are the main agents of lipolysis early in fermentation.

During sausage ripening, a significant portion of the protein is hydrolyzed.[131] Nonprotein nitrogen increases by about 20% in smoked sausages.[43] Experiments by Verplaetse et al.[189] and others[41] indicate that tissue proteases (in particular, cathepsin D) are the main agents of proteolysis early in fermentation.

Cathepsin D is activated at pH values of about 5.0 and produces peptides that are then further metabolized by lactobacilli[126,127] and catalase-positive cocci. During air drying of sausages, molds growing on the surface also hydrolyze proteins and catabolize amino acids. This is easily recognized by the ammonia smell of ripening rooms and the resulting products. The intensity of flavor of such sausages was found to be proportional to the degree of proteolysis.[60]

Amino acids not assimilated by the microflora are further metabolized, both in smoked[43] and air-dried sausages. Most branched-chain volatiles probably originate from amino acids.[66] The ammonia and the amines formed, in conjunction with lactate oxidation at the surface and an increase in osmolarity, account for the rise of the pH that is observed during drying of sausages. This rise is particularly marked in unsmoked products where molds and yeasts colonize the surface and degrade not only proteins but also lactic acid. These products obviously taste quite different to the smoked "sour" sausages. A proper selected mold culture on the sausage surface also keeps oxygen away from the sausage, provides a "microclimate" that favors the correct drying of the product, and prevents the establishment of undesired molds.

Yeasts also affect color and flavor when added to the sausage mixture.[32,58,125,156] These effects may be due to their oxygen-scavenging activity, but possibly also may be due to other by-products of fermentation and/or to their action on lipids; particularly high amounts of carbonyls (especially long-chain aldehydes) have been found in yeast-ripened sausages.[100]

Amino acids and their nitrogen-containing degradation products may contribute to the taste of the sausage but less to the aroma, because few if any nitrogen-containing volatiles have been found in fermented sausages.[13,35] Various esters (mainly ethyl esters) are found among the volatiles of fermented sausages,[35] and to contribute to salami odor. The data of Stahnke[175] suggest staphylococci (eg, *S. xylosus*) as main agents.

19.5 STARTER CULTURES

19.5.1 History

Use of starter cultures for meat fermentation has been reviewed recently.[68,83] The roles of, and benefits from, different species of microorganisms available as starter cultures for sausage fermentation have been outlined in the previous paragraphs.

The interest in starter cultures arose parallel to the trend toward industrial production, short ripening times, and standardization of product properties. In the US, the key role of lactobacilli in sausage fermentation was recognized early [Jensen and Paddock, US patent no. 2,225,783, Dec. 1940]; however, early attempts to introduce *Lactobacillus* strains as starters failed because suitable strains did not readily survive lyophilization. *Pediococcus cerevisiae*, now classified as *Pediococcus acidilactici*, proved suitable for the manufacture of products like summer sausage or thüringer cervelat,[36,37] which are fermented at temperatures above 30°C. *P. acidilactici* can be easily stored in a lyophilized or frozen form and is still widely used as starter in the US. In Europe, sausage makers were faced with the problem that a rapid lactic acid fermentation suppressed the organisms containing catalase and nitrate reductase, with consequent faults in color and flavor. Hence, they regarded lactobacilli as spoilage rather than beneficial agents, and a *Micrococcus* strain was first introduced as a starter culture by Niinivaara.[130] This strain (M 53) was selected because it rapidly reduced nitrate, improved color and flavor, and inhibited undesired microorganisms.[140] Further work by Niinivaara's group in Finland[136] eventually led to the development of starters containing a combination of a "Micrococcus" (later identified as *Staphylococcus carnosus*[165]) with *Lactobacillus plantarum*, the latter strain being superior to *Pediococcus cerevisiae* in sausage fermentation at 20° to 22°C. Early attempts to improve sausage manufacture and quality by using "atypical lactobacilli" (for example, *L. sakei*, and *L. curvatus*) as starters were of limited success,[148] even though these strains dominate in virtually all traditional European-style sausages. However, strains suitable for sausage ripening were selected later and became commercially available.

19.5.2 Selection Criteria for Starter Cultures

Selection criteria for starter cultures for meats have been summarized by Jessen[83] and Knauf.[93] Under the conditions (eg, pH, a_w, temperature, concentrations of curing agents) prevailing during fermentation, they must perform their desired activities at appropriate rates and after a lag as short as possible. They must be suitable for large-scale cultivation and preservation by freezing and lyophilization. They must not be pathogenic and must neither form toxins nor antibiotics. Moreover, they should not form compounds conveying unpleasant sensory properties. During *in vitro* screening of lactic acid bacteria for suitability as starter cultures, strains forming CO_2 from hexoses, amines from amino acids, volatile sulfur compounds from sulfur-containing amino acids, slime, or large amounts of hydrogen peroxide are rejected. Laboratory and pilot-plant sausage fermentation tests are then done to select strains of lactic acid bacteria, or any other microbial group, that form pleasant aromas and flavor notes.

Technological criteria for selection of mold starters include competitiveness on the sausage surface (ie, in the presence of salt, curing agents, and lactic acid, and at temperatures of about 15°C), adhesiveness to casings, white or slightly yellowish color (ie, no formation of conidia during sausage drying), and formation of appropriate aroma and flavor. Strains fulfilling these requirements belong to the species *Penicillium nalgiovense* (biotypes 2, 3, 6) and *Penicillium chrysogenum.* Any potential mold starter must be carefully tested by chemical and biological assays for the absence of mycotoxins and antibiotics, bearing in mind that most strains of *P. nalgiovense* and *P. chrysogenum* form the mycotoxin cyclopiazonic acid[49] and many strains form the antibiotic penicillin.[6] Mintzlaff and Leistner[124] selected a strain of *P. nalgiovense* as a starter culture, and later investigations[53,79] showed that other strains of *P. nalgiovense* and *P. chrysogenum* are likewise beneficial to the appearance and flavor of air-dried sausages.

19.5.3 Choice and Application of Starters

The necessity and choice of starters depends on the type of fermented sausage to be produced. Use of lactic acid bacteria is important for the safe production of rapidly fermented semi-dry sausages, whereas at low ripening temperatures (in particular, below 18°C), the effect of starters is small, and excellent fermented sausages have been (and still are) manufactured without addition of starter cultures or reinoculation of finished sausages. Species of which strains are commercially available as starters have been listed in Table 19-2. *P. acidilactici* is only effective at fermentation temperatures above 25°C as used in the manufacture of US-type summer sausage; in the temperature range between 20° and 25°C, its rate of acid formation is too slow to be of much benefit, and cultures containing *L. sakei* strains tend to have the fastest rates and to be most competitive. Acidification rates by *P. pentosaceus*[74,85,163] and *L. curvatus*[98] depend greatly on the culture used, and may be equal to or higher than those of *L. plantarum* and *L. pentosus. P. pentosaceus* is often stated to form a "milder" aroma.[83] *P. pentosaceus* and *L. plantarum* are not very effective in suppressing large numbers of lactobacilli present in the raw material.[33,119] However, excessive lag phases after inoculation of the mix may abolish the advantages of using a culture with a high acidification rate. With this in mind, a "starter sausage" containing a strain of *L. curvatus* is produced centrally and distributed frozen, mainly in The Netherlands.[96] Under these conditions, care must be taken to ascertain sufficient activity of desired acid-sensitive bacteria. At any rate, it is essential to follow the instructions of the starter manufacturer, and not arbitrarily combine different starters, sugars, and fermentation temperatures.

As pointed out earlier, use of starters containing catalase-positive cocci is particularly useful for products prepared with nitrate, and for products to have a long shelf life. The addition of sufficiently high levels (10^6–10^7 g^{-1}) of nonpathogenic *Staphylococcus* or *Micrococcus* (*Kocuria*) spp. largely eliminates the need to allow these bacteria to multiply in the product by keeping the pH in the permissive range (>5.3). Strains of *S. carnosus, S. xylosus,* and *Micrococcus* (*Kocuria*) *varians* are commercially available. Because of its requirement for oxygen, *M. varians* is less competitive in sausages than are the staphylococci, but it has a higher activity of nitrate reductase at 15°C.[93] Whether or not a yeast (*Debaryomyces hansenii/Candida famata*) or a culture containing *Streptomyces griseus*[46] is useful depends very much on the aroma note desired. Mold starters are generally recommended for the manufacture of mold-ripened products.

Starter cultures are distributed frozen or freeze dried on suitable carriers. Frozen cultures generally have a shorter lag phase although this is somewhat less critical than in dairy fermentations that are to be finished after hours rather than days. An even distribution of the culture in the mix is important to ensure homogenous fermentation.[89] Some sausage manufacturers resuscitate lyophilized cultures for several hours before use, but none of them propagate commercial starters *in vitro* any further.

Most commercial preparations contain a combination of lactic acid bacteria with nonpathogenic staphylococci or micrococci. For slower fermentations and/or products with a long shelf life, preparations containing less or even no lactic acid bacteria are also used. Defined cultures have shown to be useful for the manufacture of a wide spectrum of traditional sausage varieties and have replaced, to a large extent, so-called "back-slopping" methods where a fermented sausage from the previous batch is used to inoculate the following batch. The disadvantage of this method is that desired catalase-positive cocci will tend to become "diluted out," and strains with undesirable metabolic properties may be selected.

Lücke et al.[113] provided information on the use of starter cultures for sausage fermentation in various European countries. The data (summarized in Table

19-3) reflect the differences between the countries with respect to the predominant product types and the level of standardization and automation achieved by the processors.

Lactic acid bacteria and staphylococci are also available as starter cultures for the ripening of raw hams. If injected into the meat, these cultures are useful if cuts with high pH (6.0–6.2) are to be processed into raw hams.[82] In traditional manufacture, however, there is little benefit from these cultures because temperatures and a_w values on the surface of meat are too low. Certain strains of *Halomonas* or *Vibrio* may be useful for the curing of hams in nitrate-containing brines,[121] but the small market, problems with culture preservation, and the costs of safety testing appear to have prevented their marketing so far. For similar reasons, no Gram-negative bacteria are available in starter cultures for sausage fermentation, even though Petäjä[139] was able to improve the aroma of fermented sausages by combining an *Aeromonas* strain with *Lactobacillus plantarum.*

19.6 APPLICATION OF HACCP TO THE PRODUCTION OF FERMENTED MEAT PRODUCTS

It is generally agreed that the microbial safety of food can be most effectively achieved if the manufacturer follows the principles of the Hazard Analysis Critical Control Point (HACCP) concept as outlined in Chapter 58. Accordingly, there is legal pressure to introduce HACCP. Unfortunately, there is a lot of confusion resulting from inconsistent use of terms, mistranslations, and misconceptions. In agreement with the Guidelines of the Codex Alimentarius Commission, this section defines only those steps in production as "critical control points" (CCP) where loss of control may increase the health risk for the consumer, and where monitoring is feasible. HACCP complements "good hygienic practice," ie, general hygiene rules for facilities, equipment, and staff as detailed in various laws, directives, and codes of practice; for example, in the EU directives or Codex Alimentarius Commission recommendations on meat hygiene. For references and detail descriptions of Good Hygienic Practice and HACCP, see Chapter 58.

Only microbial hazards are dealt with in this section, although chemical hazards (eg, drug residues in the meat) or physical hazards (eg, splinters of bones or metals in the meat) are also relevant. However, chemical hazards (except for polycyclic hydrocarbons from smoke, and nitrite and/or nitrosamines) must be kept under control by good animal husbandry and slaughterhouse practices; the physical hazards are not specific to fermented meats.

19.6.1 Survival and Growth of Foodborne Pathogenic Bacteria in Meat Fermentations

Salmonella *and* Yersinia enterocolitica

During properly controlled sausage fermentation, the Enterobacteriaceae count remains constant and

Table 19-3 Use of Bacterial Starter Cultures for Sausage Fermentation in Various Countries

	% of Production		
Country	*Mold Fermented*	*Industrial*	*Use of Bacterial Starters*
Denmark, Sweden, Norway, Finland, Netherlands	0	>95	lactic acid bacteria for virtually all products; Gram (+) cocci less frequently
Germany, Belgium	10	85	for *ca.* 80% of production (less for slow-fermenting traditional types and for "fresh," not aged products)
Austria	30	80	as for Germany
Switzerland	60	50	as for Germany
France	95	90	for ca. 50% (less for slow-fermenting traditional types)
Italy	80	60	for 20% to 30% of production
Spain	65	70	for 20% to 30% of production ("backslopping" still common)
Greece, Portugal	<20	>80	for <30% of production ("backslopping" still common)

Source: Adapted with permission from F.K. Lücke et al., Starter Culture Development, in *Processing and Quality of Foods, Vol. 2: Food Biotechnology: Avenues to Health and Nutritious Products,* P. Zeuthen et al, eds., pp. 2.11–2.36, © 1990, Aspen Publishers, Inc.

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then decreases. Factors that may favor the growth of Enterobacteriaceae during sausage fermentation, and thereby increase the risk of salmonellosis, include a high initial water activity; a high initial pH value; a low concentration of fermentable carbohydrates; low numbers of lactobacilli in the fresh sausage mixture; the use of nitrate or of very low levels of nitrite as the curing agent[73,170]; and high ripening temperatures.

In Germany, some outbreaks of salmonellosis have been traced to the consumption of "Frische Mettwurst" (fresh, spreadable sausages). To retain their "fresh" consistency and "meaty" taste, such sausages have high water activity, are often manufactured with little, or no, sugar or acidulant (GdL), and are subjected only to a fermentation at 20° to 25°C for 1 or 2 days. This is sufficient for cured color formation but does not always suppress the growth of Enterobacteriaceae. In such sausages, increasing the amount of added salt to at least 2.5% (w/w) and addition of appropriate lactic acid bacteria, together with some fermentable carbohydrate or small amounts of GdL, is most effective for the control of Enterobacteriaceae including salmonellae.[166]

During drying, Enterobacteriaceae including salmonellae and yersiniae are slowly inactivated. Reported rates for *Salmonella* destruction during sausage ripening differ markedly from less than 1 log[160,176] to 3–4 log in smoked dry products with low final pH.[172] Likewise, inactivation rates for *Yersinia enterocolitica* during sausage fermentation vary between zero and 5 log, mainly depending on rate and extent of acid formation and drying.[8,90] For European-style smoked products, a reduction of numbers of salmonellae by 1 or 2 logs[75] is in agreement with the observation that salmonellae occur much less frequently in dry sausages than in ground meat or fresh, only slightly fermented raw sausages.[15,166] Nevertheless, outbreaks of salmonellosis from semi-dry or dry sausages have also been reported. In one of them,[106] a processor had combined very low input of nitrite (as common in Italian-type sausage formulations) with a fermentation temperature above 25°C (as common for US-style summer sausages). Another outbreak affected consumers (mainly children) who had eaten dry salami sticks.[34] Apparently, the salmonellae present in the raw material did not multiply during fermentation but survived the drying process, and the infective dose was small because the salami sticks were eaten as snacks by infants and young children.

Enterohemorrhagic Strains of Escherichia coli (EHEC)

Two severe outbreaks of hemorrhagic colitis from fermented sausages occurred in western states of the US and in Australia.[7] The infective dose of the causative agent, enterohemorrhagic strains of *Escherichia coli* (EHEC), is generally low, and multiplication during sausage fermentation is not required to cause disease. Moreover, inactivation during sausage ripening may be slow: reductions by 1 to 5 logs were observed, mainly depending on pH, water activity, ageing time, and temperature,[51,62,94,177] and possibly also as a result of acid adaptation of the cells.[107] Fortunately, the prevalence of EHECs in meat appears to be low and restricted to beef and lamb.[16] However, as long as no effective control measures on the farm or at meat inspection level exists, it is advisable not to include beef- or lamb-containing raw sausages with short ripening times into the diet of small children (the main risk group for hemorrhagic colitis and its life-threatening sequela disease, acute renal failure).

Staphylococcus aureus

Staphylococcus aureus is frequently found in raw meat and fermented sausages, but generally only at low levels. Only if allowed to grow to cell densities above 10^7 g^{-1}, does it form levels of enterotoxins sufficient to cause illness. Growth of this organism has been studied during ripening of various types of raw sausages. The bacterium is little affected by salt and nitrite, but it is a poor competitor under anaerobic conditions, at low pH values and low temperatures (see Chapter 47). It may grow at high fermentation temperatures during the lag phase of the development of lactic acid bacteria and may reach high numbers in the outer layers of the sausages.[10] Niskanen and Nurmi[132] and Metaxopoulos et al.[122,123] showed that the initial pH value and the initial activity of the lactic acid bacteria are critical for the control of *S. aureus*. If sausages are fermented at a temperature no higher than 25°C for 2 to 3 days, and the initial count of *S. aureus* is below 10^4 g^{-1}, the risk of enterotoxin formation is low,[75] even if the sausages are not smoked.[76,122,123] However, fermentation temperatures up to 43°C are common in the manufacture of smoked, semi-dry sausages in the US. Under such conditions, only a rapid pH drop in the sausages early in fermentation ensures inhibition of *S. aureus*.[143] Consequently, the American Meat Institute, in 1982, specified the maximum time t_{max} (hours) allowed in "good manufacturing practice for fermented dry or semi-dry sausage" to reach pH 5.3.[9] The equation is $t_{max} = A/(T\text{-}15)$ where T is the maximum fermentation temperature (°C) and A is a coefficient equalizing 720 for fermentation temperatures between 24° and 32°C, and 560 and 500 for fermentation temperatures above 32°

and 40°C, respectively. Apparently, the use of this rule combined with the introduction of appropriate process controls and starter cultures significantly reduced the incidence of outbreaks of *S. aureus* food poisoning in the US due to the consumption of fermented "summer sausages."

The effect of various formulations and ripening conditions on *S. aureus* in unsmoked sausages fermented at temperatures common in Europe has been studied by Hechelmann et al.[74] If fermented at 23°C, a reduction in pH to 5.3 within 2 to 3 days also turned out to be critical in these products. This could be achieved by adding lactobacilli or *Pediococcus pentosaceus* as starters, with 0.3% of a rapidly fermentable sugar. It was also important to adjust the initial water activity to above 0.955, and to use ageing temperatures below 15°C until the water activity had decreased to below 0.90. At fermentation temperatures of 15°C, no growth of *S. aureus* was observed, irrespective of acid formation.

Listeria monocytogenes

Because of its wide distribution on meat and its ability to grow at low temperatures and relatively low water activity (>0.93), *Listeria monocytogenes* may be expected to be a hazard to the consumer of fermented sausage. However, there is no epidemiological evidence for the involvement of fermented sausages in outbreaks of listeriosis, and little if any growth was observed during sausage fermentation under conditions resembling commercial practice,[52] even in undried sausages[88,164] and in mold-ripened salamis.[17,78,153,184] Conditions controlling salmonellae and *S. aureus* were found also to prevent growth of listerias. However, to make sure that listerias do not grow on the surface of mold-ripened sausages, Rödel et al.[153] recommended lowering the ageing temperature for Italian-type salami to 8° to 10°C.

During sausage ripening, listerias die only slowly. The use of lactobacilli that form a listeriocidal bacteriocin as starters reduced the *Listeria* count early in fermentation by about 1 log.[14,56,78,164]

Spore-Forming Bacteria

Raw sausage mixtures may contain considerable numbers of *Bacillus* spores of which spices are a major source. Many *Bacillus* spp. show proteolysis and/or lipolysis in appropriate nutrient media. However, they fail to multiply and to cause spoilage during sausage fermentation, even if high numbers of facultatively anaerobic species and/or species capable of growing at low a_w are inoculated into raw sausage mixtures, and if faulty ripening conditions are used.[129] The authors concluded that *Bacillus* spp. are controlled in fermented sausages by a combination of low water activity, low pH, and absence of oxygen. The experience of manufacturers of fermented sausages in Germany also indicates no need to select spices with low numbers of *Bacillus* spores.

There is no practical or epidemiological evidence that clostridia may grow and form toxins during ripening and storage of fermented sausages. Botulinum toxin was only detectable if a sausage prepared with no glucose added was inoculated with about 10^4 spores of proteolytic strains of *Clostridium botulinum* per gram, heated to 58.3°C core temperature and subsequently kept at high temperature (27°C) for extended time in which the pH did not fall below 5.5.[23] This is far from any commercial practice. Growth and toxin formation by *C. botulinum* (proteolytic or nonproteolytic strains) is even less likely in "European-type" fermented sausages.[112,134] *C. botulinum* and other clostridia are controlled by the combination of pH and water activity in fermenting sausages, irrespective of nitrite addition, and do not represent a hazard for fermented sausages.

19.6.2 Control of Other Biological Hazards

Molds and Mycotoxins

Leistner and Eckardt[105] and Leistner[101] listed those mycotoxins that have been found on fermented sausages experimentally inoculated with toxigenic molds. Aflatoxins and other *Aspergillus* toxins were only found if such sausages were aged at elevated temperatures and relative humidities that are very unlikely to prevail in commercial practice.[2] If, as in normal practice, sausages are dried at temperatures of 15°C or below, toxinogenic aspergilli are outgrown by penicillia and *Scopulariopsis* spp.[81] Among the *Penicillium* toxins, small amounts of cyclopiazonic acid and verrucosidin and, possibly, citreoviridin and rugulosin may be formed in the outer layer of the sausage under these conditions.[105,161] Even though the levels found in experimentally inoculated sausages were small and restricted to no more than a few millimeters below the surface, growth of all undesirable molds on fermented sausages should be prevented, not only to eliminate the risk of mycotoxin and antibiotic formation, but also to avoid defects in appearance and flavor. The following preventive measures should be taken:

1. The surface of products to be mold ripened should be inoculated with a suitable nontoxic mold strain and the products dried at temperatures below 15°C.

2. On other fermented meats, mold growth may be inhibited by appropriate control of temperature and RH during ageing, and/or by surface treatment with smoke, permitted antifungal agents, or waxes. Antifungal smoke constituents as well as acetic and propionic acids are volatile and cannot safely prevent mold growth during extended storage. After fermentation, products may be dipped in solutions of antifungal agents such as potassium sorbate or natamycin (pimaricin). However, natamycin is an antibiotic that is also used in treatment of humans. Hence, its use for preservation of fermented sausage is restricted in many countries.
3. Packaged products to be stored at elevated temperatures for long periods (eg, snack sausages) should be dried to 22% moisture (corresponding to a water activity of 0.82 or less) and stored in the absence of oxygen. This results in the complete suppression of the growth of molds and a delay of rancidity.[183]

Viruses

The potential viral hazards in meats and the fate of viruses during meat processing have been reviewed by Cliver[24] and McKercher et al.,[120] respectively (see also Chapter 52). Viruses already present in livestock at the time of slaughter normally do not affect humans. Foot-and-mouth-disease virus is acid sensitive and likely to be inactivated during sausage fermentation.[47,138] The causative agents of African swine fever, swine vesicular disease, and hog cholera are inactivated only slowly during sausage ripening and are sometimes detectable even after 3 months. Therefore, fermented meats should, like unprocessed meats, not be exported from areas where these diseases prevail. There is no epidemiological evidence that fermented sausages are a major vector of viruses pathogenic for humans; however, enterovirus, echovirus, and poliovirus retain their infectivity during fermentation and drying of sausage. Hence, avoiding the handling of raw materials and fermented meats by hands contaminated by human feces is critical to prevent transmission of human pathogenic viruses via fermented sausages.

Parasites

Some parasites (eg, protozoa, nematodes, tapeworms) may escape detection during inspection of the animals and their meat at the slaughterhouse (see Chapter 51). Fortunately, they are inactivated by the low a_w values in dry sausages and dry hams. For example, *Toxoplasma* was rapidly inactivated during manufacture of fermented sausage,[173] and there is no epidemiological evidence of fermented meats being involved in the transmission of disease caused by protozoa. To inactivate 100–800 *Trichinella* larvae per gram fresh mixture, German-type sausages (eg, salami, cervelat, Teewurst) had to be ripened for 6 to 21 days and to attain a water activity (a_w) of 0.93–0.95. The low pH contributes to inactivation because in products of higher pH such as Genoa-type salami and raw hams, lower a_w values were required.[22,109] However, it is safe to state that fully dried meat products (a_w below 0.90) will not contain any viable protozoa or larvae of helminths, even if prepared from contaminated meat. If the presence of the larvae cannot be excluded by meat inspection, semi-dry and undried fermented sausages must be made from meat that has been stored frozen, or be heated to 58.3°C (137°F), as stipulated by US regulations.[9]

Biogenic Amines

Fermented foods may contain considerable amounts of amines, which may not only be precursors of nitrosamines but also elicit migraine or allergy-like symptoms in humans. Mean and median values of about 100 mg and maximum values of about 300 mg histamine kg^{-1} dry weight have been reported,[162,185,186,194] with a trend to higher levels in aged, mold-ripened products. Such levels, as well as the reported concentrations of other biogenic amines (eg, up to 1,000 mg kg^{-1} dry weight of tyramine) are unlikely to elicit scombroid-type poisoning or migraine-like symptoms in healthy individuals having normal activity of monoamino oxidase.[115] However, the published data also show that it is possible to produce high-quality fermented sausages with very low levels of biogenic amines. Hence, a better understanding of the factors affecting formation of biogenic amines during sausage fermentations is necessary.

The rate of amine formation is limited by the rate of protein degradation by tissue enzymes and, in particular in mold-ripened products, microbial enzymes.[48] Hence, more precursors (eg, peptides, amino acids) must be expected in meat stored for long time periods[185] or in mold-ripened products. Conditions enhancing the activity of acid-sensitive bacteria appear to increase the risk of biogenic amine formation.[118,162] By using good-quality, fresh raw material, appropriate starters, formulations and ripening conditions, formation of biogenic amines during sausage ripening may be minimized. Remarkably, certain strains of psychrotrophic lactic acid bacteria found on meat (eg, *Carnobacterium* spp.; strains of *L. curvatus*) are also capable of decarboxylating certain amino acids.[117,181] Hence, it is necessary to screen potential starter cultures for absence of the ability to decarboxylate amino acids.

There appears to be no formation of nitrosamines during meat fermentations,[97,133] even though the precursors (secondary amines and nitrite) may be present. However, severe heating at low a_w favors nitrosamine formation. Therefore, fermented meats should not be fried, and the residual levels of nitrite and nitrate should be minimized. There is no technological or microbiological reason for the addition of more than 150 mg sodium nitrite per kg to even the most unstable product (fresh, undried sausage), and excess nitrite is reduced to ammonia by the sausage microflora, usually leaving less than 10 mg $NaNO_2$ per kg of finished product.[192]

19.6.3 Risk Assessment

Using the information given in Section 19.6.1 and considering exposure to the agent, severity of the disease, and epidemiological data, the main risks to the consumer of fermented sausages are salmonellosis, infections caused by enterohemorrhagic *Escherichia coli,* and *Staphylococcus aureus* enterotoxicosis. Mycotoxins, viruses, parasites, and biogenic amines should not be forgotten, but risks to the consumer of fermented meats appear to be small, and preventive measures have already been discussed in Section 19.6.2.

19.6.4 Preventative Measures To Control Foodborne Pathogenic Bacteria

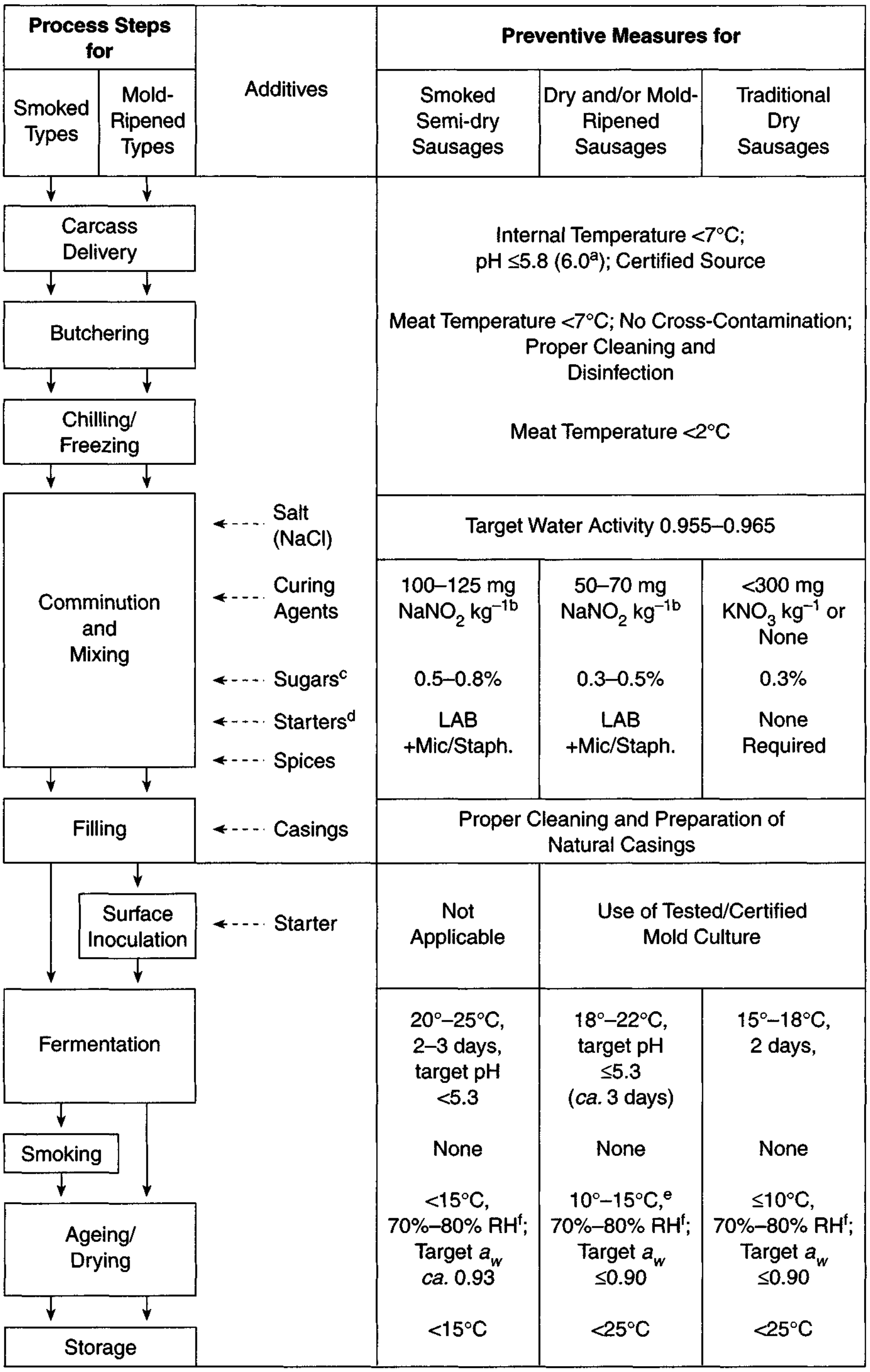
Figure 19-2 illustrates the main process steps critical for the control of growth of *Salmonella enteritica, Staphylococcus aureus,* and *Listeria monocytogenes* during sausage ripening, and the preventive measures to be taken. It is important to note that "lowering" of one "hurdle" may be compensated for by "fortifying" another hurdle.[102] For example, one may increase the initial water activity of the sausage mix within certain limits (as sometimes done for the manufacture of low-fat, low-sodium products) if the rate of acid formation is increased or the fermentation temperature lowered. This must be taken into account when setting up HACCP systems for the manufacture of fermented meats.

As long as there are no effective methods available to eliminate EHECs from livestock (particularly from cattle and lamb), the most important places for reducing EHEC contamination of the meat used for fermented sausage manufacture are during the skinning and evisceration of cattle and lamb. This is because at these stages, the risk of contamination of meat by fecal material is high. Accordingly, manufacturers of fermented sausages should take great care to buy their raw material from abattoirs with good hygienic practice. In addition, meat and meat products must not be touched by hands contaminated by human feces.

19.7 MECHANISMS AND CONTROL OF SPOILAGE OF MEATS DURING AND AFTER FERMENTATION

A delay in acid formation not only increases the risk of growth of pathogens but also leads to elevated numbers of Enterobacteriaceae causing off-odors and off-flavors. Improperly cleaned natural casings may contaminate the surface of the sausages with Enterobacteriaceae and inhibit drying of the product, thus favoring growth of these bacteria. The most frequent cause of spoilage of fermented sausages is undesired metabolic activities of lactobacilli. Use of excessive amounts of fermentable sugars, in conjunction with insufficient drying and elevated ageing and/or storage temperature, results in high levels of lactic and acetic acids, sometimes even in hole formation and swollen packs due to CO_2 production by heterofermentative lactobacilli.[72]

Lactobacilli are also involved in the formation of gray or greenish discolorations of sausages; this is related to hydrogen peroxide formation. Peroxides attack heme compounds and polyunsaturated fatty acids and thus lead to color and flavor defects, particularly if soft, improperly stored fatty tissue has been used.[72] Individual species and strains differ widely in their ability to accumulate and to destroy hydrogen peroxide.[30,114] Strong producers are often found among strains of *L. sakei* and *L. curvatus* because these species, unlike *L. plantarum,* possess flavin-dependent oxidases.[87] However, many strains of lactic acid bacteria from meats contain catalase activity when grown on myoglobin- or heme-containing media,[135] or heme-independent "pseudocatalase" (Table 19-2).[50] Thus, net hydrogen peroxide production in meat may be low even in the absence of catalase-positive acid-sensitive microorganisms. Nevertheless, practical experience indicates that oxygen



Process Steps for		Additives	Preventive Measures for		
Smoked Types	Mold-Ripened Types		Smoked Semi-dry Sausages	Dry and/or Mold-Ripened Sausages	Traditional Dry Sausages
Carcass Delivery	Carcass Delivery		Internal Temperature <7°C; pH ≤5.8 (6.0[a]); Certified Source		
Butchering	Butchering		Meat Temperature <7°C; No Cross-Contamination; Proper Cleaning and Disinfection		
Chilling/ Freezing	Chilling/ Freezing		Meat Temperature <2°C		
Comminution and Mixing	Comminution and Mixing	Salt (NaCl)	Target Water Activity 0.955–0.965		
		Curing Agents	100–125 mg $NaNO_2$ kg^{-1b}	50–70 mg $NaNO_2$ kg^{-1b}	<300 mg KNO_3 kg^{-1} or None
		Sugars[c]	0.5–0.8%	0.3–0.5%	0.3%
		Starters[d]	LAB +Mic/Staph.	LAB +Mic/Staph.	None Required
		Spices			
Filling	Filling	Casings	Proper Cleaning and Preparation of Natural Casings		
	Surface Inoculation	Starter	Not Applicable	Use of Tested/Certified Mold Culture	
Fermentation	Fermentation		20°–25°C, 2–3 days, target pH <5.3	18°–22°C, target pH ≤5.3 (*ca.* 3 days)	15°–18°C, 2 days,
Smoking			None	None	None
Ageing/ Drying	Ageing/ Drying		<15°C, 70%–80% RH[f]; Target a_w *ca.* 0.93	10°–15°C,[e] 70%–80% RH[f]; Target a_w ≤0.90	≤10°C, 70%–80% RH[f]; Target a_w ≤0.90
Storage	Storage		<15°C	<25°C	<25°C

[a] In exceptional cases; adjustment of sugar addition and fermentation conditions may be necessary.

[b] With max. 500 mg Na-ascorbate/kg.

[c] Rapidly fermentable by the starter used. 0.8% should be used if meat pH is significantly above 5.8.

[d] LAB, lactic acid bacteria; Mic/Staph., *Micrococcus/Staphylococcus*. Starters must be active at the fermentation temperature selected.

[e] For mold-ripened products, temperature should be lowered to about 10°C after 2 days of fermentation.[153]

[f] RH, relative humidity. Adjustment depending on pH and a_w of sausage (see text).

Figure 19-2 Preventive Measures To Control Growth of *Salmonella enteritica*, *Staphylococcus aureus*, and *Listeria monocytogenes* during the Manufacture of Fermented Semi-dry and Dry Sausages. *Source:* Reprinted from F.K. Lücke, Fermented Sausages, in *Microbiology of Fermented Foods*, 2nd ed., Vol. 2, B.J.B. Wood, ed., pp. 441–483, © 1998, Aspen Publishers, Inc.

should be excluded from raw sausage mixtures as far as possible, and enough peroxide-destroying activity should be present during fermentation. This is achieved, for instance, by slowing down the rate of acid formation by lowering the fermentation temperature, and/or by the addition of appropriate starters (ie, lactic acid bacteria that do not accumulate peroxides, with suitable catalase-producing microorganisms such as *Micrococcus* and *Staphylococcus* strains).

After fermentation, undesired mold growth and/or oxidative changes in the lipid fraction (rancidity) limit the shelf life of most semi-dry or dry sausages.

19.8 RECENT AND FUTURE DEVELOPMENTS

In 1988–89, the present author collected the opinions of various experts from industry and research on desirable improvements of sausage fermentation.[113] Priority was given to shortening ageing times (ie, obtaining better flavor in less time) and prolonging shelf life by inhibiting rancidity, while acceleration of acid production ranked low. However, in the last few years, suppression of pathogens (in particular enterohemorrhagic *E. coli*) and amine formation have become major issues. In addition, there is continuous interest in making microbiological and chemical changes during sausage ripening more predictable, a prerequisite for standardization and automation of the manufacture of these products.

19.8.1 Novel Cultures

Possible developments in the field of starter cultures have been discussed in various reviews,[68,69,83,113] and requirements for good starters are given in Section 19.5. It must be emphasized that starter cultures are just one factor affecting quality and safety of fermented sausage and cannot make up for incorrect formulations, eg, use of rancid fat, addition of too much sugar, or incorrect ripening conditions. One difficulty is that, unlike milk and malt, meat cannot be pasteurized before inoculation without drastically changing its properties. This means that any starter culture for sausage ripening must be able to compete with the indigenous microflora of the sausage mixture. Moreover, selection and improvement of starters for better aroma in less time and long shelf life is difficult because the chemical and biochemical reactions affecting sensory properties are very complex and insufficiently understood. This limits the possibility of screening a large number of strains in model systems, as well as the prospects of tailoring strains by means of genetic engineering. However, novel starters may increase product safety, and much work has been devoted to the selection of strains antagonistic to pathogens. Bacteriocins formed *in situ* by lactic starter cultures may contribute to the inactivation of *Listeria monocytogenes* (see Section 19.6.1), and *Penicillium nalgiovense*[59] and meat lactobacilli[20] have been engineered to lyse *Staphylococcus aureus* by means of lysostaphin. However, prospects of using such strains are limited. For example, bacteriocins from lactic acid bacteria are insufficiently stable in meat systems, have little if any effect on Gram-negative bacteria, and bacteriocin-resistant variants of target bacteria may arise.[111] Moreover, *Staphylococcus aureus* can be controlled well by the use of existing technology, making it difficult to explain to the public and to regulatory bodies the necessity of using genetically engineered organisms. For further discussion of bacteriocins and their use see Chapter 12.

The complexity of reactions leading to optimal aroma and flavor of fermented meats makes it very difficult to accelerate sausage ripening. However, selected enzymes may have an effect on sausage ripening. For example, Næs et al.[127] obtained a more intense flavor of a fermented sausage by adding a peptidase isolated from *Lactobacillus paracasei.*

19.8.2 Novel Methods of Fermentation Control

A mass of data on the behavior of pathogens is now available in electronic form,[155] and predictions may also be made on the performance of lactic acid bacteria in sausages as affected by water activity, temperature, and other parameters.[98,145] Mathematical models for sausage fermentation have been published,[41,188] and a model to predict inactivation of *Enterobacteriaceae* during sausage ripening has also been published.[11] Such tools may be helpful in optimizing existing, and developing new, processes.

On-line sensors for various fermentation parameters (temperature, pH, relative humidity, air velocity, equilibrium humidity of the product) are available,[154] and data may be used to control the fermentation.[179] This could lead to shorter ripening time and less labor caused by manually adjusting the ripening climate or transporting the products from one chamber to another. For example, a product pH below 5.3 means that the fermentation is nearly finished, and the temperature and relative humidity in the ripening chamber should be lowered.

19.8.3 Novel Products

While there is a large variety of fermented milk products, fermentation has not been employed to any extent in the manufacture of meat products other than "classical" fermented sausages. However, as shown for bacon,[182] it may be possible to improve the safety of nonfermented meats by addition of selected lactic acid bacteria.[4,60] To date, such attempts have limited success because of sensory differences (sour taste is not tolerated in most meat products) and shortening of shelf life. Moreover, the decrease in water-binding capacity connected with lowering the pH to 5.3 is undesirable in most pasteurized meats except those that are subsequently dried, eg, "Kochsalami."[151] A better knowledge of the role of microorganisms in aroma and flavor formation may be applied to develop raw ham and nonfermented meat products with improved sensory properties. Fermentative production of aroma concentrates to be added to such meats may be an option.

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20 Fresh and Further-Processed Poultry

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CHAPTER CONTENTS

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20.1 INTRODUCTION

The term *poultry* normally includes chicken, turkey, duck, goose, and guinea fowl, although it is sometimes used to encompass species such as pigeon, ostrich, pheasant, and other game birds. Over the last 40 years, the poultry meat industry has undergone considerable expansion, and poultry now accounts for some 27% of the world's meat consumption. Chicken and turkey are the principal types of poultry meat, with chicken comprising two-thirds of the total production and an annual output of more than 33,000 million birds worldwide.[2] Production methods vary from that of the free-range village chicken of developing countries to the highly intensive systems used in the more developed world, where poultry production is one of the most technologically advanced sectors of agriculture. In many of the larger companies around the world, the operation is partly or completely integrated, and a single organization may own breeding farms, hatcheries, feed mills, growing units, and primary and further-processing plants. Over the years, there have been continuous improvements in bird growth performance and feed conversion, and considerable economies of scale in both production and processing. The latter now involves a high degree of automation and, overall, the industry is particularly efficient and progressive.

Poultry meat is perceived as cheap, wholesome, and nutritious, being high in protein and low in fat, with a favorable content of unsaturated fatty acids. Unlike pork and beef, there are no religious constraints on consumption. Also, poultry meat is easy to prepare in the home and widely utilized in fast-food establishments. In developed countries, the boom in poultry consumption has been fueled by the recent expansion in further processing, which leads to better utilization of the meat and upgrading of off-cuts or poorer cuts. It also provides portion control, convenience, variety, and relatively consistent product quality. Thus, poultry meat is now used to prepare many of the traditional products that previously were made only from red meats, as well as a variety of other items. These include cut portions, reformed roasts, rolls, escallops, grillsteaks, burgers, turkey hams, nuggets, sausages, frankfurters, salamis, bolognas, and ready meals. Both product appearance and flavor can be varied as required. With cured products, however, the degree of curing and smoking is less than that used in red meat processing, and the goal is to provide distinctive flavors rather than preservation. Product formulations vary widely in different countries and are influenced by local tastes and customs. The details are usually confidential to particular companies.

There are two main kinds of poultry muscle: white (breast) and red (leg). These have structural and physiological differences, as well as different *postmortem* pH values. Breast muscle has a pH value in the range 5.6–5.8, and leg muscle is pH 6.1–6.4. Both types of muscle are relatively susceptible to microbial spoilage when stored in the unfrozen state. The shelf life of raw meat depends upon the combined effects of certain intrinsic and extrinsic factors, including the numbers and types of psychrotrophic microorganisms present initially, the storage temperature, muscle pH and type, as well as the kind of packaging material used and gaseous environment of the product during storage.

Despite the healthy image among consumers, raw poultry is a common vehicle of foodborne human disease, especially from *Salmonella* and *Campylobacter*. Illnesses from each of these agents have an estimated annual incidence in the United States (US) alone of up to 4 million cases.[18] The organisms are carried asymptomatically in the intestines of a varying proportion of all healthy birds, and they frequently become contaminants of processed carcasses. Other foodborne pathogens that are often present on carcasses include *Clostridium perfringens*, a common intestinal organism, and *Staphylococcus aureus*, which is carried on the skin and in the nasopharynx of some birds and may also be acquired from the colonization of processing equipment.[76] Generally, both of these organisms occur in only low numbers, and the meat is unlikely to become hazardous unless it is temperature abused and extensive multiplication occurs. Chicken and turkey are the main types of poultry incriminated in human food poisoning, but their respective involvement[18] does not reflect the consumption figures, which are always lower for turkey. Unlike chicken, turkey is often cooked as a whole carcass, particularly for receptions and other large gatherings and on festive occasions. Large carcasses may be cooked a day in advance of consumption and, due to their size, they take a relatively long time to thaw, cook, cool, and reheat.[18,123]

Although *Salmonella* is recognized as the most important foodborne pathogen associated with poultry meat, the extent to which poultry is responsible for human salmonellosis is presently unknown, because most cases are sporadic and cannot be traced to source. Data from outbreaks in the United Kingdom (UK), however, suggest that 20% to 35% of reported cases could be associated with poultry consumption.[119] During the period 1989–1991, 11% of 392 general outbreaks of salmonellosis were linked to poultry meat and *S. enteritidis* was incriminated in 48% of these.[129] Throughout much of the world, the recent rise in human salmonellosis has been paralleled by an increase in the incidence of *S. enteritidis* in poultry flocks. There is also concern about the possible presence of *S. typhimurium* DT104, which is particularly pathogenic for humans and shows multiple resistance to antibiotics, including some of those used in human medicine. In a study of human illness from this organism, chicken from butchers' shops, restaurants, and food take-away establishments was among the risk factors identified.[145] *Salmonella* contamination of poultry carcasses varies widely between countries.[141] Table 20-1 shows the results of a number of surveys published between 1990 and 1994, with an incidence of positive samples between 4% and 100%. It has been reported[118] that giblets (heart, liver, gizzard, neck) are more frequently contaminated with salmonellas than the carcasses themselves. From 1987 onward, the most common serotype isolated in the UK was *S. enteritidis* PT4, which was responsible for much of the increase in human salmonellosis over the period in question.[119] These results contrast sharply with the situation in Sweden, where salmonellas have been virtually eliminated from poultry meat through a combination of governmental controls and voluntary measures taken by producers. However, the cost of such a program has been considerable and was estimated to be five times that of the British system.[109] Also, Sweden has a much smaller, less complex poultry industry and a colder climate, all of which may contribute to the very low incidence of salmonella-contaminated poultry meat. Other Scandinavian countries with a low incidence are Finland and Norway.

Enteric disease in man from *Campylobacter* is mainly due to *C. jejuni* and *C. coli* and, again, the majority of cases are sporadic, with outbreaks being relatively uncommon. Of all the reported outbreaks of foodborne disease in Europe during 1990 and 1991, only 1.1% were due to *Campylobacter*.[37] Data from sporadic cases suggest that handling and preparation of chicken and poultry liver, and the consumption of undercooked chicken are particular risk factors[119]

Table 20-1 Reported Salmonella Contamination in Poultry and Poultry Products[18,119,141]

Year	*Country*	*Type of Product*	*No. of Samples*	*Percent Positive*
1990	India	dressed broilers	50	4
1990	Netherlands	chicken portions, livers	81	54
1991	UK	chicken (hospital kitchen)	214	45
1991	US	post-chill chicken	57	11
1991	US	retail broilers	36	17–50
1991	India	fresh and frozen chicken	?	100
1991	UK	retail chicken	292	48
1992	US	post-chill chickens	158	72
		cut portions	154	77
1992	Germany	fresh poultry	238	51.7
1992	US	post-chill broilers	560	40.8
1993	US	pre-chill broilers	500	21.8
1993	Germany	poultry products	?	12.7
1994	UK	retail frozen chicken	281	41
		retail chilled chicken	281	33

while, in the US, consumption of chicken, Cornish game hen, and, to a lesser extent, turkey meat have been linked to human campylobacteriosis.[29,44] In most countries, poultry meat is frequently contaminated with campylobacters, and up to 100% of processed carcasses may be positive for these organisms. Counts from skin can exceed 10^4 g^{-1}, but levels of contamination are often reduced during processing to about 10 g^{-1} or 10^3 to 10^6 per carcass.[49,83,142] The organisms could be isolated from breasts, wings, thighs, and abdominal cavities, indicating a general distribution over the carcass. Some reports show a lower contamination rate for frozen chickens in comparison with chilled carcasses, but this may be influenced by the method of isolation and whether or not damaged cells were recovered. Studies suggest[124,134] that campylobacters may survive on frozen chicken for at least 3 to 4 months.

Food poisoning from *C. perfringens* is also associated with poultry, especially turkeys consumed during the festive season. Spores of this organism can survive normal cooking regimes.[123] Although campylobacteriosis and illness from *S. aureus* is declining, outbreaks involving poultry and poultry products are well documented. In the case of *S. aureus*, the cooked meat is usually contaminated by an infected food handler.[47] *Listeria monocytogenes* is a common contaminant of raw poultry and about 60% of chicken carcasses may carry the organism in low numbers.[81,111] It can also be found on cooked poultry in some cases, despite stringent measures to prevent contamination. Cooked chicken nuggets and cook-chill chicken have been implicated in sporadic cases of foodborne listeriosis.[60,61] In the US, turkey hot dogs were the vehicle in one instance, and the same strain of *L. monocytogenes* was isolated from the patient, from hot dogs kept in the patient's refrigerator, and from unopened packs of the same brand of product obtained from a local shop.[11] Other psychrotrophic pathogens are also common on raw poultry meat, especially *Aeromonas* spp. and *Yersinia enterocolitica*,[141] although their public health significance in this context is not entirely clear. Both types of bacteria grow readily on poultry meat under chill conditions, but there is little evidence of any hazard from these organisms when raw poultry products are stored normally. The strains of *Y. enterocolitica* are not usually those associated with human yersiniosis.[27] Verocytoxin-producing *Escherichia coli* O157:H7 has been found in poultry in the US, where strains were isolated from 1.5% of 263 retail samples of chicken and turkey.[31]

20.2 SOURCES OF PRODUCT CONTAMINATION WITH HUMAN PATHOGENS AND SPOILAGE ORGANISMS

20.2.1 The Large-Scale, Live-Bird Production System

The possibilities for contamination of carcasses in the processing plant are strongly influenced by the microbiological status of the live bird and this, in turn, is related to husbandry practices and the efficacy of hygiene control measures throughout the production chain. The stages in a typical chicken operation are shown in Figure 20-1. Elite stock are family groups of in-bred lines carrying certain desirable genetic traits, eg, body conformation, meat yield, and disease resistance. Grandparent stock are bred from the best of elite lines to provide parent birds, which produce the chicks that will be reared for meat production. Broilers are immature birds that are slaughtered at about six weeks of age or less, according to weight requirements. In addition, hens at the end of their useful laying life are also sent for slaughter, usually at specialized slaughterhouses. The meat obtained in this case is used mainly in further processing, ie, in cooked products, soups, etc.

Both breeders and broilers are normally kept on litter (wood shavings or straw) in housing where the environmental temperature and ventilation rate can be controlled. Flock size varies with the type of bird, parent flocks comprising 4,000–5,000 birds and broilers 10,000–20,000 birds or more. Stocking densities are up to 20 birds m^{-2}. Free-range systems represent a small sector of the industry in developed countries and have the disadvantage that there is no control of environmental conditions, while the ground area may need to be changed periodically to prevent any build-up of disease. Hygiene control measures are most stringent at the top of the production pyramid and specific precautions are taken to protect elite and grandparent flocks from disease. For example, no visitors are allowed and staff are required to take a shower and don special protective clothing before entering the houses. The eggs from these flocks are hatched in separate, dedicated hatcheries.

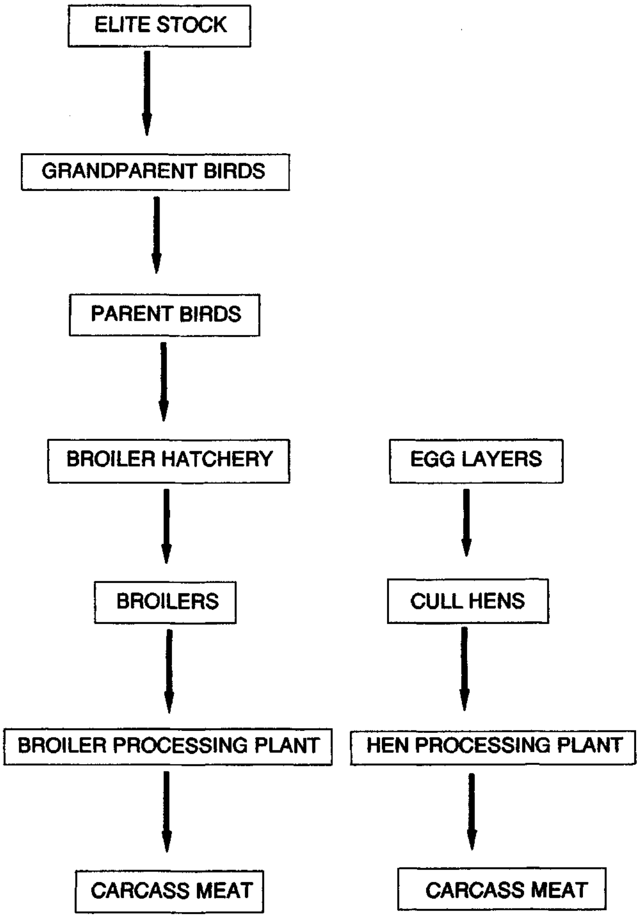

Figure 20-1 Typical Chicken Production Chain.

For all birds, the hatchery represents the area of greatest disease risk and high standards of hygiene are always necessary. The layout of the building should be such that there is adequate separation of "clean" and "dirty" operations and the air flow must pass from clean to dirty areas. Requirements for hygienic operation include easily cleanable surfaces, with no dust traps. Rodents, flies, and other pests must be suitably controlled. Staff are required to wear protective clothing and to exercise good personal hygiene. There should be appropriate facilities for cleaning and disinfecting the egg-collecting vehicles. A regular program of microbiological testing should be used to monitor the efficacy of the cleaning regime throughout the hatchery.

Only visually clean eggs should be incubated and, on arrival at the hatchery, the eggs may be fumigated with formaldehyde. Alternatively, they may be dry cleaned, washed, or dipped in a sanitizing solution. Eggs can be stored for up to seven days before incubation. A further fumigation treatment is often used before the eggs are incubated in "setters," where they remain for 18 days. Then, they should be examined to ensure that embryos are developing and transferred to hatchers for a further three days. Facilities do not normally allow segregation of eggs from different flocks. During incubation, contaminated egg shells and other debris, such as chick fluff, can serve as sources of infection in the incubator and airborne infection can also occur. Once in the egg, salmonellas can multiply rapidly and infect the developing embryo. This will then become a source of infection for other chicks as they hatch. Thus, every attempt must be made to exclude salmonellas from the hatchery environment.

Broiler chickens, or meat birds of other species, may acquire salmonella infections from three main sources: (1) breeding stock via the hatchery, (2) the rearing environment, and (3) contaminated feed. *Salmonella enteritidis* is currently the most common serotype causing human illness in various countries[119] and eggs can be contaminated with this organism, either internally or externally by the time they are laid. Alternatively, eggs may become contaminated after lay by penetration of the shell. *S. enteritidis* is an invasive serotype with an apparent predilection for the reproductive tract of poultry. Infection of the oviduct appears to be a major source of egg contamination[27] thereby causing vertical transmission from parent to progeny. Other invasive serotypes include *S. typhimurium* and *S. virchow*, but the extent to which they are transmitted vertically among poultry flocks is less clear. Most salmonellas are confined to the intestines of infected birds and excreted in the feces. In broiler flocks, both these and the invasive serotypes may spread horizontally from birds that are shedding the organisms to those that are not. Once introduced, infection may be spread via drinking water and feed that rapidly become contaminated in the house. Potential vectors include wild birds, rodents, insects, domestic animals, and humans.

Rodent proofing of poultry houses requires particular attention, while all farm personnel must wear protective clothing and visitors to the farm should be kept to a minimum. Domestic pets should be excluded at all times. From the point of view of hygiene control, all birds should be removed from the house at the end of the rearing period (the "all-in, all-out" principle). In practice, thinning of flocks may occur when birds of certain weights are required before the normal slaughter age. This could result in the introduction of salmonellas and other foodborne pathogens because the houses must be opened up to collect the birds, thus exposing the remaining birds to infection.

When the house is finally emptied, spent litter and feces are usually removed from the farm, after which the equipment and internal environment must be thoroughly cleaned and disinfected. Cleaning of houses is particularly difficult for premises with earthen rather than concrete floors, although they do not always harbor salmonellas. Empty houses should be "rested" before introducing a new crop of birds to allow a natural die-off of any disease agents present. The ideal period is about three weeks, but shorter times become necessary when there is commercial pressure to utilize rearing facilities to the full.

During rearing, the third potential source of salmonella infection is the feed and this is due mainly to the use of contaminated ingredients in which salmonellas may survive processing, or recontamination of feed that occurs at the mill or on the farm. Even one salmonella g^{-1} of feed is sufficient to produce infection.[46] Ingredients of animal origin are known to be prone to salmonella contamination; these include meals from fish, meat and bones, feathers, and blood. In consequence, UK legislation, for example, requires that all processed animal protein, whether home produced or imported, is tested for salmonellas. However, no such requirement applies to vegetable proteins or other ingredients, despite the fact that virtually all ingredients have been shown at various times to be contaminated with salmonellas.[115,152] Nevertheless, measures are taken at feed mills to safeguard the final product, including the application of heat treatment, which is a necessary part of the pelleting process used for broiler feed. The efficacy of the treatment depends upon temperature, time, moisture, and initial levels of contamination, and may involve the use of expander technology or an extrusion process. Recontamination of pelleted feed can occur during air cooling, unless the air being drawn in is filtered. After processing, it is essential that the feed is kept dry because areas of dampness may lead to multiplication of surviving organisms. Water can enter the milling environment via condensation, leaking pipes and roofs, etc. Trucks used to transport feed should be dedicated to this task and be cleaned and disinfected between loads.

The use of ionizing radiation is a possible alternative to heat treatment for poultry feeds, but the high cost is prohibitive. Short-chain fatty acids, such as formic and propionic, are sometimes added to feed and have the advantage of protecting it against recontamination during distribution and storage. The acids have been shown to reduce the incidence of salmonella infection in the birds,[45,54] but appear to have little effect in dry feed. It is only when the feed becomes hydrated after consumption by the birds that significant antibacterial activity is likely to occur. Acid-treated feed should be given throughout the rearing period because the acids have no beneficial effect once the birds have become infected.[45] Many of the salmonellas isolated from feed and feed ingredients are not among the more important causative agents of human salmonellosis. However, there are well-established instances where an association has been evident, eg, the presence of *S. agona* in fish meal in 1970 and subsequent isolation from poultry and human cases of illness. Although it is thought that *S. enteritidis* may have been introduced into poultry flocks via contaminated feed,[115,117] this organism is only rarely isolated from finished feeds or ingredients.

20.2.2 Biological Control Measures in Poultry Production

With the recent increase in human food poisoning from *S. enteritidis*, new approaches to salmonella control have become necessary in the poultry industry. One such approach arises from the recognition that the newly hatched chick is highly susceptible to salmonella infection because it is produced in a strictly sanitized environment, has no contact with parent birds, and is slow to develop an intestinal microflora that could successfully compete with ingested pathogens.[101] Thus, Nurmi and his colleagues in Finland[101,112] developed a simple means of establishing an adult-type gut flora in chicks by dosing the birds orally with suspensions or anaerobic cultures of gut contents from adult, salmonella-free individuals. In this way, the treated chicks became resistant to an oral challenge of about 10^6 salmonellas/bird. The protective effect, which is usually termed "competitive exclusion" (CE), is not significantly influenced by the breed, strain, or sex of bird, but depends upon the introduction of viable bacteria, especially anaerobes.

Although attempts have been made to identify the protective bacteria and develop treatment preparations of known composition, these are generally less protective than anaerobic cultures or suspensions of gut content and, in time, tend to lose their protective capability.[130] Therefore, the most widely used treatment preparations are based on the original approach and are essentially complex, undefined cultures of caecal material. It is necessary, however, to screen them rigorously to ensure the absence of all relevant avian and human pathogens. In practice, CE treatment is used in two ways. The first treatment is for newly hatched chicks that are spray inoculated in the hatchery. The birds are wetted on the upper part of the body and ingest the treatment organisms while preening themselves. The second method is applicable to older birds, usually breeding stock, that are already salmonella carriers and require antibiotic therapy to eliminate the infection. The therapy is followed by administration of a CE preparation via the drinking water to prevent reinfection. The effect of CE treatment on salmonellas entering the gut is largely to prevent their growth, so that the organisms are gradually eliminated. However, the exact mechanism of protection is unclear and is likely to be influenced by factors such as pH and Eh; inhibitory substances such as H_2S, bacteriocins, volatile fatty acids, and deconjugated bile acids; competition for receptor sites and nutrients; and local immunity. For *in vivo* conditions, these factors may be interdependent.

Under laboratory conditions, protection has been demonstrated against at least 10 *Salmonella* serotypes, including both invasive and noninvasive strains, and first becomes evident within a few hours of administering the gut-flora preparation. The degree of protection obtained may be reduced in chicks subjected to stress due to high or low environmental temperatures or feed and water deprivation.[146] On the other hand, most antimicrobial growth promoters and anti-coccidial agents have little or no effect on the protective process. CE treatment has been used routinely in Sweden for a number of years, with no adverse effects on bird health or growth performance.[148] In relation to *Salmonella*, it is seen as an important part of the broiler control program.[149] In trials carried out in other countries, especially The Netherlands, the treatment has reduced both the number of salmonella-positive flocks and the proportion of birds within flocks that become infected (Table 20-2). In the UK, commercial use of CE is mainly confined to breeder chicks at present. Where CE treatment is to be part of the control strategy, salmonella-free chicks are a necessary starting point because the treatment is a prophylactic and cannot be expected to eliminate an existing salmonella infection. The use of CE must also be combined with good husbandry hygiene because, while the protective flora is becoming established, the chicks will remain susceptible to infection. Also, any infected chick is a potent source of infection for its cohorts and, with little microbial competition in its intes-

Table 20-2 Field Trials on Competitive Exclusion (CE) Treatment of Broiler Chicks

	Incidence of Salmonella-Positive Birds at Slaughter[a]			
	CE Treated[b]		*Untreated*	
	No. Tested	*% Positive*	*No. Tested*	*% Positive*
Flocks	143	14.7	141	24.1
Birds	14,400	0.9	14,099	3.5

[a]Based on caecal examination.

[b]Treatment material: intestinal homogenate from specific pathogen-free birds, spray inoculated in the hatchery.

Source: Data from E. Goren, et al., Reduction of Salmonella Infection of Broilers by Spray Application of Intestinal Microflora: A Longitudinal Study, *Veterinarian Quarterly*, Vol. 10, pp. 249–255, © 1988.

tines, will commonly shed 10^7–10^8 salmonellas g^{-1} of feces for other chicks to acquire. Such a large challenge is capable of overcoming the protection offered by CE treatment.

Following the upsurge in poultry infections with *S. enteritidis*, there has been increasing interest in vaccine development because vaccines have been used in the past in controlling the invasive poultry pathogens *S. gallinarium* and *S. pullorum*. Research has centered on live, attenuated, and dead vaccines and some are available commercially. A new inactivated vaccine, which specifically aims to reduce vertical and horizontal transmission of *S. enteritidis*, is being used in the UK. When broiler parent stock are vaccinated with this preparation, it is claimed that chicks show passive immunity for at least 21 days. Although vaccination could have a part to play in controlling specific, invasive salmonellas, there may be little, if any, effect on serotypes that are confined to the alimentary tract, where their reaction with the immune system of the bird is largely unknown. As with CE treatment, successful use of vaccination depends upon other control measures being used simultaneously, especially a high standard of husbandry hygiene.

20.3 THE PROCESSING PLANT

20.3.1 Outline of Processing

In industrial countries, poultry are brought to the processing plant in trucks that are specially designed to hold large numbers of birds in crates or modules that can be readily unloaded. After varying periods of time in the arrival area, the birds are transferred individually to a continuously moving system of shackles, on which they are hung manually by the legs. The birds proceed usually to an electrical stunning machine that either renders them unconscious or actually kills them. This is followed by the bleeding stage, in which the neck is partially severed, either manually or by means of an automatic, rotating knife blade. After bleeding, the birds are scalded by immersion in hot water in order to loosen the feathers. The water temperature varies from 50° to 63°C, depending on the bird species being processed and the ultimate type of product (chilled or frozen). Low scald temperatures are required for birds being sold as chilled products, because this avoids the skin being damaged during defeathering (see below). Feathers are removed from the birds via a series of on-line plucking machines. The machines contain banks of counter-rotating domes or discs with flexible rubber "fingers" mounted on them. These remove the feathers by their scouring action. The next stage is usually to remove the head, which is done by an automatic head puller, and then the feet by means of an automatic foot cutter. Next, the birds are transferred to the evisceration line, where both edible and inedible viscera are removed, either by an entirely manual process in the smaller slaughterhouses, or with the use of automatic machinery. Several different machines are involved, each concerned with a separate operation. Both during and after the evisceration stages, carcasses are spray washed to remove any visible spots of blood or feces and then chilled, either by water immersion or air blast chilling, both of which are continuous processes in the larger premises. In low-throughput establishments, water chilling may be carried out in slush-ice tanks and air chilling in a cold room. Finally, carcasses are weighed, graded, and packaged prior to freezing or chill storage, or they may be transferred to a portioning operation. Although individual processing plants differ in points of detail, the basic process is virtually the same worldwide.

20.3.2 Microbiological Problems in Processing

When birds arrive at the processing plant, they carry a large microbial load and many different kinds of microorganism occur on the skin, among the feathers, and in the alimentary tract.[108,143,144] The process of converting the live bird into an oven-ready product leads to removal of a high proportion of the organisms but, during processing, further contamination can occur at any stage, especially during scalding, plucking, and evisceration, but also from aerosols, processing equipment, and the hands of operatives. Initially, the predominant organisms are Gram-positive rods and micrococci, but the final product has a largely Gram-negative flora, including *Flavobacterium* spp., Enterobacteriaceae, *Pseudomonas* species, and *Actinobacter/Psychrobacter* spp.[5,7,70]

The microbiological problems of poultry processing are similar to those encountered in the slaughter process used for red meat animals, but some features of poultry processing are unique and have possible microbiological consequences. Over the years, for example, the rate of processing has steadily increased and many processing plants are producing 6,000 carcasses or more per hour on a single line. With high-rate processing, the carcasses on the line are very close together and cross-contamination occurs readily. Also, the carcass remains whole throughout the process, which means that the viscera must be removed through a relatively small opening. This operation often leads to gut breakage and contamination of the carcass with gut contents. Furthermore, it is sometimes difficult to clean the abdominal cavity of an entire carcass effectively. Unlike other meat animals, the skin is not removed during processing and is already contaminated with microorganisms when the birds arrive for slaughter.

Conditions during breeding, hatching, and rearing of the birds are conducive to the spread of minority organisms such as *Salmonella*. The problem continues during the transportation of birds to the processing plant, especially when cleaning and disinfection of crates and vehicles is not done properly, so that there is flock-to-flock transmission of foodborne pathogens, even before birds are transferred to the processing line.[14,122] The extent to which cross-contamination occurs subsequently depends upon the conditions of processing, as described below, and the measures taken to control plant hygiene. Because the process is so rapid and the small carcasses cool relatively quickly, there is little opportunity for multiplication of mesophilic bacteria on the carcass itself. However, some microbial growth is possible on certain items of processing equipment and this can lead to increases in carcass contamination. Growth of psychrotrophic bacteria on wet surfaces of equipment occurs more readily and is likely to account for contamination of carcasses with listerias[51,104] as well as spoilage organisms.

20.3.3 Microbial Attachment to Carcasses

Even before the attachment phenomenon was studied in any detail, it was appreciated that the washing of poultry carcasses would normally remove only a fraction of the organisms present on the skin, and even the most efficient post-evisceration spray washer would achieve no more than a 10-fold reduction in count. Also, many organisms located on the skin appear to be largely unaffected by the heating involved in immersion scalding or the use of super-chlorinated process water. An understanding of the mechanism of microbial attachment to carcasses might be expected to facilitate the development of measures to remove and destroy microbial contaminants. The attachment of microorganisms to poultry skin is unlikely to be a simple matter because of the nature of the skin surface and the fact that changes occur as birds pass through different stages of the process. For example, there is a major change in the surface structure as a result of defeathering, which leaves many holes in the skin that can trap bacteria. The skin itself is a complex surface, as shown by electron microscopy, which has revealed an irregular topography with many microscopic channels.[71,135] After chilling, an undefined layer of material has been observed at the surface and this appears to support microbial growth during storage of the carcass.

The initial studies on the attachment of bacteria to chicken skin were based on empirical observations and involved immersing carcasses in buffered saline containing known concentrations of the test organisms.[96,97,98] Only those cells that remained on the skin after washing for 3 minutes were regarded as "attached." It was found that the attachment of *Escherichia coli* increased with time of immersion and was directly related to the number of cells present. Conditions of pH and temperature influenced the rate of attachment. While attached organisms were not easy to remove, those present in the film of liquid over the skin surface could be washed away by the washing technique. It is generally recognized that organisms present in the liquid film are likely to become attached to the skin in the course of time.[66,98,135] The early studies suggested that the possession of flagella was essential for an organism to become attached to poultry skin. Little or no attachment occurred with nonmotile organisms such as a *Klebsiella* spp. or *Lactobacillus brevis*. However, later work[69] showed that nonmotile bacteria comprise >70% of the microflora of processed chicken carcasses, thus casting doubt on the suggestion that flagella are necessarily involved in bacterial attachment. Subsequently, no difference was found between flagellated and nonflagellated bacteria in attachment capability and attachment was unaffected by the presence of fimbriae.[65,66]

The electron microscopical work[71,135] shed new light on the problem and showed that, when the skin was wetted, swelling occurred, as water was absorbed. The swelling appeared to trap bacteria that were already located in crevices and channels in the skin and increased their inaccessibility. Skin swelling is not, however, the whole story. When skin was inoculated without significant wetting, bacterial attachment still occured.[66] Clearly, the situation is complex, and there is likely to be more than one mechanism by which microorganisms become attached to the skin or entrapped in it. Removal of attached bacteria is not easy to achieve in the context of poultry processing. One approach involved the use of 4% D-mannose,[28] which prevented attachment by *Pseudomonas aeruginosa* and also removed attached cells, but this would be too costly for commercial application. Washing in saline helped to remove many attached cells,[136] while use of a surfactant may also be beneficial by changing the surface tension of the water film.[66] Use of trisodium phosphate as a chemical rinse at the end of processing is said to act partly by removing a microscopical layer of surface fat from the skin, which allows attached bacteria to be washed away.[23]

20.3.4 Effects of Processing on Carcass Contamination

It is important that birds are starved prior to slaughter so that the intestines are not distended, thereby reducing the risk of gut breakage during evisceration. The exact period of feed withdrawal is a matter for debate. While at least 3 to 4 hours is necessary for this purpose,[42] there is also support for periods of 9 to 10 hours to ensure that the crop is completely emptied. The disadvantage of extending the withdrawal period is that the feces become more fluid and therefore the chances of cross-contamination during transportation would appear to increase. In any case, transporting birds from the farm to the processing plant tends to increase levels of contamination on the outsides of the birds. Over a 5-hour period, a 10-fold increase in *E. coli* has been observed.[108] During the journey, the skin and feathers are either contaminated from the bird's own droppings or, if the flooring on each tier is perforated, birds below may become cross-contaminated. These problems are exacerbated under wet weather conditions, which tend to spread fecal material among the birds.[150]

It is often assumed that the stress involved in transportation increases the likelihood that birds will become shedders of *Salmonella*. Experiments on simulated transport stress, involving crowding, movement, and chilling, showed that stress increased the proportion of salmonella shedders from 33% to 54% within 4 hours, but the level declined thereafter.[121] An earlier study, with longer holding periods was unable to demonstrate any increase in the proportion of birds shedding salmonellas, although the organisms could be isolated more frequently from the body cavity and liver, suggesting that systemic infection had occurred.[125] Inadequate cleaning and disinfection of vehicles and transport crates after use appears to be a common problem. In one study, 46% of birds from a salmonella-free broiler flock became contaminated after being loaded into contaminated crates.[122] With the older type of system in which the crates are fixed to the vehicle, cleaning must be done manually by hosing with chlorinated water, for example, and insufficient time may be devoted to this task.[82] For crates that can be cleaned individually, away from the transporter, an automated system that involves a soaking period and machine washing, together with the use of a sanitizer, is preferred.

In modern processing plants, advances have been made in relation to the hanging of birds on the processing line. Crate design has been improved and access to the birds by the operatives has become easier. Nevertheless, unloading inevitably causes a degree of struggle and wing-flapping, which scatters dust and microorganisms, including any foodborne pathogens present.[106,153] It is necessary for this part of the operation to be physically separated from the remainder of the process.

Stunning of the birds, or killing by electrocution, is usually carried out with a waterbath stunner, and the head should be only partly immersed to avoid inhalation of contaminated water. In practice, some of the birds do inhale a small amount of water, which may penetrate into the lung cavity.[41] This is unavoidable at present, although the problem can be minimized by ensuring a constant overflow from the stunner bath. It would be eliminated entirely by the use of gas stunning. Cutting the neck to bleed out the carcass is not associated with any severe microbiological problem, although, with high-rate processing, there is no opportunity for heat treatment of the knife blade between individual birds. Cross-contamination can be reduced, however, by irrigating the rotating blade continuously with a stream of chlorinated water.[82]

After bleed-out, carcasses pass into the scald tank, where the hot, circulating water removes large numbers of microorganisms. It has been estimated that each carcass contributes about 10^9 organisms to the water, as it passes through the system.[92] Some of the older literature[63,137] indicates that birds could inhale water during the scalding process, leading to contamination of internal organs. This should not occur in birds that have been properly stunned and bled. Inevitably, the scald water becomes heavily contaminated with microorganisms but conditions are such that, after an initial build-up, the number of viable organisms will remain relatively constant over the working period.[85] Fresh water is added continuously to replace that removed by the birds and some processors maintain an overflow, which is required by law in the US. This overflow is generally too small to reduce microbial numbers in the water. The most important factor affecting microbial levels is the scald temperature.

Temperatures used for scalding poultry depend mainly on the required type of end-product. For the fresh, chilled market, a "soft" or semi-scald at 50°–52°C is necessary because this ensures that the cuticle is not loosened and subsequently damaged during defeathering, and it avoids any severe discoloration of the skin when carcasses are air chilled. The problem does not arise with carcasses that are water chilled and frozen and that acquire a surface layer of fine ice crystals. In this case, a "hard" or sub-scald at 56° to 63°C is used because feather removal is facilitated at the higher temperatures. At 50°C, bacterial survival is much greater in the scald water, and organisms such as *Salmonella* may persist long enough to cause cross-contamination of carcasses.[21] For example, a $D_{52°C}$ value of 150 seconds was reported for a strain of *Salmonella typhimurium*,[94] whereas the corresponding value at 62°C was only 8 seconds. Even at the highest temperatures used, there would be little effect on bacterial spores, including those of *C. perfringens*,[92] whereas highly heat-sensitive organisms such as *Pseudomonas* spp. are readily destroyed, even at 50°C.[4] Because of the large number of microorganisms being added to the scald water, this stage in poultry processing is inevitably an important site of cross-contamination.[72,94] Alternatives to conventional immersion scalding include spray or steam scalding and combined scalding and plucking, in which hot water sprays are used. Spray scalding has a higher water requirement and can have adverse effects on the appearance of carcasses. The use of steam is unsuitable for carcasses being produced for the chilled-product market because of damage to the outer cuticle.

A different approach to controlling microbial levels in scald water is to continuously add an antimicrobial chemical. Chlorine would be ineffective because of rapid inactivation, but 0.1% acetic acid markedly reduced $D_{52°C}$ values for *Campylobacter jejuni, S. newport,* and *S. typhimurium*.[105] Microbial survival is also related to the pH value of scald water, which is consistently around 6.0 because of dissociation of ammonium urate that is present in poultry feces. Experimental addition of sodium hydroxide or sodium carbonate increased the pH value to 9.0 ± 0.2 and reduced the $D_{52°C}$ value for *S. typhimurium* from 34.5 to 1.25 minutes.[52] These findings apply only to microorganisms in the water. Generally, organisms attached to the skin of the birds are more protected from the effects of heat and chemicals. Even at a concentration of 0.5%, acetic acid had little effect on carcass contamination, and the same was true for pH adjustment of scald water.[12,53]

Mechanical removal of the feathers from freshly scalded carcasses involves two particular problems. One is due to extensive aerial dispersion of organisms in the vicinity of the machines; the other involves microbial growth in the machinery and can lead to measurable increases in carcass contamination. Cross-contamination has been studied with the aid of a readily identifiable "marker" organism, a strain of *E. coli* K12, that can be detected on a highly selective isolation medium. Experiments have shown that, during defeathering, the "marker" can be transmitted to at least 200 birds following one that had been deliberately inoculated.[78,140] Organisms appear to spread mainly from the surfaces of the birds and not from extruded feces.[94] It is important that the scalding and plucking processes are carried out in a well-separated area of the processing plant to minimize the opportunities for microbial dispersion.

The defeathering machines are necessarily sited close to the scald tank so that feathers can be removed while the carcasses remain warm. Inside the machines, however, conditions are warm and moist and therefore favorable for microbial growth. Also, the rubber "fingers" harbor microorganisms and are particularly difficult to clean and disinfect properly. In addition, the "fingers" soon become worn and cracked, thus allowing organisms to penetrate below the surface, where they are relatively well protected. Surprisingly, the organisms that appear to flourish best under these conditions are staphylococci, especially *S. aureus* and *S. sciuri*.[76,77] The former, in particular, is not noted for its ability to compete with other types of bacteria. In this context, *S. aureus* has been studied extensively and some strains are known to persist in the defeathering machines for months or even years, because routine cleaning and disinfection are unable to eliminate them.[76] Such strains appear to be "endemic" to the processing plant in question. The organisms tend to be more resistant to chlorine than other strains and often show an unusual clumping morphology, together with the formation of extracellular slime.[15,75] It is thought that these properties may account for the greater chlorine resistance. The ultimate origin of "endemic" *S. aureus* remains unclear, although strains are most likely to be brought into the processing plant by the birds, albeit in low numbers. While most strains of *S. aureus* from poultry do not produce any detectable enterotoxins, some isolates from defeathering machines have been shown to produce types C and D toxins.[76]

Growth of staphylococci in defeathering machinery sometimes yields counts of *ca.* 10^7 cfu $swab^{-1}$ and increases levels of carcass contamination. The plucking process can also increase the proportion of birds contaminated with salmonellas.[19,88,99] At present, there is little that can be done to control the microbiological problems associated with defeathering, although the more modern machines are easier to clean and disinfect. A radical new design would be needed to overcome the problems described above.

Feather removal is more difficult in the case of ducks. The birds are scalded at 60°C, machine plucked, and then immersed in molten wax at *ca.* 90°C, which is hardened by subsequent immersion in cold water. The wax is stripped from the birds by hand or machine, thus removing the fine feathers. After use, the wax is melted again and recycled. The high temperature treatment appears to have a beneficial effect on the microbial quality of the final product, and water-chilled ducks were found to carry very low numbers of coliform bacteria.[10] For all types of poultry, it is considered advisable to pass the birds through a spray washer after the plucking process is complete.

Prior to evisceration, the head and feet are removed from each carcass; then the birds pass through a series of machines, each of which carries out a specific operation leading to removal of the viscera. In low-throughput processing plants, the same operations may be carried out manually. For both types of system, there are opportunities for carcass contamination to increase and for cross-contamination to occur. With manual evisceration, the extent of these problems depends upon the care taken by operatives. In fully automated plants, the machines must be set carefully and adjusted as required. The type of equipment used is also an important factor. Some machines cause breakage of the intestines and hence contamination of carcasses with gut contents. This is because the machines do not adjust automatically to the varying size of individual birds. Once the viscera have been exposed, the birds are ready for inspection and then removal of edible and inedible parts. However, new methods of evisceration and giblet harvesting are now available. These involve immediate separation of intestines and giblets from the carcasses and transfer of the viscera to a separate, parallel line for official inspection. This approach avoids much of the problem of carcass contamination, because the viscera are no longer in contact with the bird. Depending on the type of equipment used, some degree of cross-contamination is likely to occur through contact with machinery, implements, and the hands of operatives and has been demonstrated previously with the use of "marker" organisms.[78,131,140] Some items of equipment, such as the head puller, are known to be a source of salmonella contamination, although the problem can be minimized by continuously spraying the functional parts with water containing at least 40 mg l^{-1} of free available chlorine.[3]

Modern poultry meat production is a relatively wet process because water sprays are commonly used in the eviscerating machines, and carcasses may be spray washed at more than one stage in the evisceration process. In those countries that permit the use of chlorine, concentrations of 20–50 mg l^{-1} or more of available chlorine may be employed in the washers. Carcasses are finally spray washed, when in the "oven-ready" form, to ensure that all visible soiling from spots of blood or feces has been removed. Mechanical washers clean both the outside and inside of the bird by lowering a spray nozzle into the body cavity at the same time as high-pressure jets wash the outer surface. The cleaning process also removes some of the microbial contaminants present, but the effect is limited due to microbial attachment to the skin. Removal of microorganisms is best when carcasses are washed as soon as possible after contamination and before attachment can occur.[100] With a "marker" strain of *E. coli*, it has been shown that spray washing only at the end of evisceration reduced neither the proportion of carcasses that acquired the "marker" nor the numbers of organisms being transferred.[77] In this study, the water contained 20 mg l^{-1} of free available chlorine. While the use of chlorine has little direct effect on carcass contamination, it does control the build-up of microorganisms on equipment and destroys any spoilage bacteria that may be present in the plant water supply. *Pseudomonas* spp. are more resistant to chlorine than many vegetative bacteria and may not be eliminated during normal water works treatment.

In high-throughput processing plants, scalding, plucking, evisceration, and washing of carcasses are complete within a matter of minutes, and thus there is little loss of body heat within this period. Carcass temperatures above 30°C can be expected prior to chilling. Prompt and efficient chilling of the bird is essential to prevent growth of mesophilic pathogens and to limit multiplication of psychrotrophic organisms such as spoilage pseudomonads. There are two main types of chilling systems, one involving immersion of carcasses in cold water and the other requiring the use of cold air, with or without wetting the birds to enhance the rate of cooling through evaporation (evaporative cooling).

All poultry chilling is essentially a two-stage operation, in which the first stage precedes grading and packaging and aims to chill the product below 10°C to avoid any problem from microbial growth. Then, the bird is either graded and packaged as a whole carcass or used to prepare cut portions, after which the product is either frozen or chilled to 0° to 2°C. Water immersion chilling is a continuous process and carcasses move through one or more large tanks of water, to which ice or chilled water are added, as required. The water is kept in a state of constant agitation to facilitate cooling of the carcasses. The agitation also removes a proportion of the contaminating microorganisms. Because organisms are continuously being added to the chill water, it is important to prevent build-up over the working day by controlling water usage and temperature. Adequate use of fresh water is essential to the cooling process and also prevents the water from reaching a temperature at which considerable microbial growth could occur. Using a mathematical model designed to study the interaction of factors involved in water chilling,[58] it was shown that microbial numbers in the water would be unlikely to exceed 10^5 ml^{-1} and could be substantially less, when chilling systems are suitably controlled. Observations on commercial systems supported this conclusion.[13,73] In the chilling systems commonly used, carcasses are moved through the water, either in the same direction as the water flow (through-flow) or against it (counterflow).

Within the European Union (EU), it is mandatory to use only the counterflow type of chiller, in which carcasses meet the cleanest water as they leave the system. EU regulations (Table 20-3) specify the amount of water to be used for different sizes of carcass; how the water should be divided between chill tanks, if more than one is used; the temperature of the water at the carcass entry and exit points; and the maximum residence time for carcasses in the first part of the system. In addition, there are specific requirements relating to cleaning of carcasses prior to chilling.

Countries outside the EU may use a different mode of operation or one that is less detailed. In adequately controlled chilling systems, the washing effect on carcasses leads to a net reduction in microbial contamination and reduces both aerobic plate counts and counts of coliform bacteria by 50% to 90%.[16,74,133] Levels of salmonella contamination may also be reduced but not, usually, the proportion of salmonella-positive carcasses. Where no reduction in count has been observed, either the chilling system was not properly controlled or carcass contamination prior to chilling was unusually high.[64] An obvious disadvantage of this method of chilling is the opportunity for cross-contamination because large numbers of carcasses are chilled together in the same tank. The problem can be minimized by superchlorination of the chill water.[73] For this purpose, it is necessary to maintain a residual of 45–50 mg l^{-1} of total available chlorine, which eliminates virtually all viable organisms from the water (Table 20-4). Continuous immersion chilling is a rapid and highly effective means of cooling small carcasses, such as those of chickens. With turkeys, some of which weigh 20 kg or more, longer periods of chilling are needed and the continuous process is followed by the use of static slush-ice tanks, which further reduce carcass temperatures and ensure a tender product prior to freezing. Growth of psychrotrophic bacteria during prolonged water chilling can be controlled by adding chlorine to the process water.

Table 20-3 Operational Requirements for Water Immersion Chilling of Poultry in the European Union

Type of Chilling System	*Counterflow*
Max. water temp. at carcass entry point	16°C
Max. water temp. at carcass exit point	4°C
Total water throughput,	
up to 2.5 kg carcasses	2.5 l/carcass
2.5–5.0 kg carcasses	4.0 l/carcass
above 5.0 kg carcasses	6.0 l/carcass
Water throughput in last tank, if more than one tank,	
up to 2.5 kg carcasses	1.0 l/carcass
2.5–5.0 kg carcasses	1.5 l/carcass
above 5.0 kg carcasses	2.0 l/carcass
Time limit for carcasses in first part/tank	30 minutes

Source: Data from European Union Poultry Meat Hygiene Directive (originally 71/118 EEC).

Chilling carcasses in cold air rather than by water immersion is used for both chickens and turkeys and may be carried out as a batch process or in a continuous air-blast system, with or without a fine water spray at the beginning of the process (evaporative cooling). Because there is no washing effect, air-chilled carcasses tend to carry more microorganisms than those that are water chilled in a properly controlled system.[21,89,138] Although relatively dry in appearance initially, the carcasses have a similar shelf life to that of water-chilled birds, when both are sold as packaged products. Air chilling is preferred for some products because there is less absorption of extraneous water, and the process is less likely to cause microbial cross-contamination. Nevertheless, mi-

Table 20-4 Control of Microbial Contamination in a Water Immersion Chilling System by Superchlorination

Chlorine[a] Concentration mg l^{-1}	*Unit 1*		*Unit 2*		*Unit 3*	
	TVC,[b] 1°C	*Coliforms*	*TVC, 1°C*	*Coliforms*	*TVC, 1°C*	*Coliforms*
0	2.7	4.0	3.8	3.7	2.6	3.1
10–15	2.7	2.9	1.9	1.2	NF[c]	1.2
25–30	1.9	0.1	NF	NF	NF	NF
45–50	NF	NF	NF	NF	NF	NF

[a] Total residual chlorine. Water usage 2.5 l carcass^{-1} (total). Samples taken at 30-minute intervals over a period of 4.5 hours. All counts are expressed as mean $\log_{10}$ cfu ml^{-1}.

[b] TVC = total viable count.

[c] NF = not found.

Source: Reprinted with permission from G.C. Mead and N.L. Thomas, Factors Affecting the Use of Chlorine in the Spin-Chilling of Eviscerated Poultry, *British Poultry Science*, Vol. 14, pp. 99–117, © 1973, Carfax Publishing Co.

croorganisms may circulate in the currents of cold air, and carcasses are often touching one another in the chiller, so that some microbial transmission is inevitable. In rare instances, carcasses or cut portions are chilled by means of cryogenic methods involving liquid nitrogen or carbon dioxide. The use of carbon dioxide "snow" for secondary chilling causes crust-freezing of the product, which is not favored in the EU. Ultimately, equilibration occurs, giving a surface temperature of *ca.* –2°C and an extended shelf life.

The grading, trussing, and packaging stages follow chilling. These involve further handling of the product, possible cross-contamination from hands and equipment, and even growth of psychrotrophs if there are delays before chill storage or freezing. The freezing process can reduce the number of viable organisms present,[113] especially those of Gram-negative genera, but Gram-positive bacteria such as *S. aureus* and bacterial spores are largely unaffected.

Chilled carcasses that are not required for the whole-bird market are used to prepare cut portions. In the larger chicken processing plants, portioning is a highly automated operation, while turkeys are generally cut by hand. In both cases, there is manipulation of the meat and exposure of new cut surfaces, with associated microbial contamination and cross-contamination. It is important that the cutting environment remains cool and dry to minimize any growth of spoilage bacteria.

20.3.5 Development of Hygienic Processing Machinery

In relation to product hygiene, the main priority in poultry meat production is to reduce the incidence of carcasses contaminated with *Salmonella* and/or *Campylobacter* species to the lowest possible level. While any degree of contamination with these and other foodborne pathogens is undesirable, the nature of the present process provides no fully effective means of removing or destroying contaminants once they are acquired by the carcasses. Future improvements in hygiene control will depend upon the introduction of new equipment, involving fresh concepts. However, although hygiene control is acknowledged to be important to equipment manufacturers, it is not the first priority and, understandably, cost-effective operation of machinery is the prime consideration. Generally, the legislation in different countries does not include any specific requirements relating to the type of equipment that processors use.

Despite the lower priority given to hygiene control, several new developments have occurred in recent years. These include multistage scalding, the new type of evisceration system referred to above, better equipment for cleaning and disinfecting live-bird delivery crates, and cleaning-in-place systems for rotating evisceration equipment. Although they are not yet used universally by the poultry industry, most of the newer machines are gradually gaining acceptance. The question arises, how effective are these kinds of equipment in reducing carcass contamination? The question is difficult to answer because old and new machines cannot usually be compared at the same time and place, using birds from a common source. However, it is claimed that counterflow, multistage scalding reduces microbial contamination of carcasses by about 60%.[154] The system has the advantage that carcasses meet progressively cleaner water during the scalding process and water in the different sections can be changed whenever required. The newer type of evisceration equipment provides more controlled opening of carcasses and transfer of the viscera to a separate, parallel line, with separation of hearts, lungs, and livers. Inevitably, the process leads to less spillage of gut contents, and therefore, lower levels of carcass contamination.

In a study of counterflow scalding operated in conjunction with a post-scald, 60°C spray wash, significant reductions in carcass contamination were demonstrated.[57] On the other hand, any benefit in reducing salmonella contamination was lost during water chilling. This underlines the need for improvements in hygiene control at all stages of the process. While improvements in processing equipment will continue to reduce the microbial load on carcasses, such developments are unlikely to lead to the removal of all contaminants or avoid the problem of cross-contamination. However, more fundamental changes in machinery design and mode of operation could well conflict with the interests of processors in maintaining efficiency, speed, and product yield.

20.3.6 Application of the Hazard Analysis Critical Control Point (HACCP) System

The HACCP concept provides a systematic means of identifying, evaluating, and controlling the microbiological hazards that arise in operations concerned with food production, processing, or handling. It also involves specifying the control criteria and establishing rapid monitoring procedures so that corrective action can be taken immediately, if control is lost. Detailed records, including those of any corrective action, are essential, and the effectiveness of the control system as a whole needs to be verified, with all possible hazards identified, when the controls are operational. Both principles and specific applications are described by the International Commission on Microbiological Specifications for Foods.[55] Particular advantages of the HACCP approach are its preventive nature, dealing with hazards before they arise, and the fact that the whole process is covered comprehensively. Thus, the system is a powerful tool for ensuring product safety, and it is a company responsibility to implement and maintain a HACCP-based control program as part of the quality assurance scheme. These activities cannot be a function of the regulatory authority, and a more appropriate role for regulators is to ensure that the HACCP system is functioning effectively (verification).

HACCP principles can be applied at all stages in the poultry meat production chain, and in the UK, for example, a risk analysis approach is increasingly being encouraged to control microbial hazards associated with rearing and slaughtering of poultry.[119] In consequence, a Hygiene Assessment System (HAS) has been introduced as a means of monitoring hygiene standards in processing plants and identifying any premises where conditions are unsatisfactory.[127] The assessment is carried out by the Official Veterinary Surgeon and must take place while the processing plant is in operation, preferably at normal line speed. The aspects covered in the assessment include antemortem handling and inspection of birds, processing conditions, personnel and hygiene practices, maintenance and hygiene of the premises, and general conditions and management of the plant. Scores are awarded in one of four categories in which "a" represents the highest standard and "d" the worst. For each section of the assessment, the component scores are added together to give a subtotal. Then, a conversion factor is applied so that the final figure more accurately reflects the impact on the hygiene status of the final product. The weighting given to the various categories is, in percentage terms, processing conditions, 45; personnel and hygiene practices, 25; antemortem handling and inspection, 10; and general conditions and plant management, 10. The emphasis given to processing conditions indicates the importance of processing equipment and its mode of operation in determining the microbiological condition of finished carcasses.

The HAS approach attempts to introduce a degree of objectivity in assessing plant performance and is proving to be of value for this purpose. It concentrates effort in those areas that are most likely to benefit product safety and provides a yardstick by which the overall performance of the industry can be judged. For individual processors, the system is a tangible inducement to attain the highest level of performance, and it could be adapted for computer-assisted control of hygiene in the processing plant. Processors who are applying HACCP principles are likely to achieve a high score in the HAS. Generic HACCP schemes for poultry processing have been described,[55,95] but these "blueprints" serve merely as guides. Experience shows that no two processing plants are identical in all respects. Consequently, processing conditions will vary from one establishment to another and each processing line requires its own hazard analysis.

Regarding the need to control gross contamination of carcasses, only chilling can be considered as a CCP1 (complete control) because it serves to prevent any problem from microbial growth. Spray washing processes are CCP2s since they only reduce carcass contamination. However, chilling is not a critical control point (CCP) for the incidence of salmonella-contaminated carcasses. In the case of water immersion chilling, chilling may even favor cross-contamination, if the process is not strictly controlled, preferably with the use of superchlorinated water. Cross-contamination can involve very small numbers of organisms. It occurs continuously throughout the process and is only partly reduced by introducing conventional control measures (see below). In this respect, the CCPs given in generic plans are unlikely to have much effect unless a decontamination step is introduced. Scalding, for example, removes large numbers of microorganisms from the freshly slaughtered birds but, in doing so, provides conditions that favor the transmission of any pathogens present. Scald temperatures of 60°C or above will destroy many of the vegetative organisms in the water, including salmonellas, but are rarely used in practice because of possible cooking effects and the need to use lower temperatures for carcasses being prepared for the chilled market. Therefore, scalding is a CCP in relation to gross contamination, because there is a net removal of organisms from the carcass surface. On the other hand, this part of the process is a significant cause of salmonella cross-contamination, and the problem is not readily avoided.

The evisceration stages pose similar problems. Careful setting of automatic machinery and resetting for flocks of different average bird size will minimize gut breakage and thus reduce fecal contamination of carcasses (CCP2). Nevertheless, many of the machines in current use cause some breakage of the intestines, regardless of the care taken in adjustment; hence, a degree of cross-contamination is to be expected.

It follows from the above that, whereas CCPs can be identified to control gross contamination, including that involving spoilage bacteria, there appear to be no CCPs in the basic process that would prevent or consistently reduce the incidence of cross-contamination with minority foodborne pathogens. This does not mean, however, that Good Manufacturing Practice (GMP) cannot combine to limit the spread of hazardous organisms when applied to the process as a whole. Examples are effective cleaning and disinfection of equipment and the processing environment after one processing period and before another, and attention to hygiene of personnel. GMP is seen as a necessary part of the HACCP program in poultry processing plants.[39] In attempts to identify and control those parts of the process where cross-contamination is known to occur, the readily identifiable enteric "marker" organism described earlier has been used to inoculate carcasses, equipment, or working surfaces.[82] The spread of the organism was then followed both before and after introducing additional hygiene control measures, which included the application of chlorinated water sprays to prevent a build-up of microorganisms on equipment, increasing chlorine concentrations in process water, and eliminating all unnecessary carcass contact surfaces. The results showed that cross-contamination could be reduced (Table 20-5) and even prevented at some points in the process.

Another study[83] investigated the effect of similar controls on the contamination of carcasses with naturally occurring campylobacters. It was found that some 95% of birds from 15 flocks yielded positive neck-skin samples after exsanguination, but processing reduced levels of campylobacter contamination by 10- to 1,000-fold and contamination was significantly lower following changes in the process to improve hygiene control. In practice, however, the reduction was relatively small and unlikely to affect exposure of consumers to these particular organisms.

20.3.7 Decontamination of Processed Carcasses

An effective and acceptable means of inactivating foodborne pathogens that are present on finished carcasses would be clearly advantageous and could provide the processor with a CCP to counteract the problem of product contamination during processing. However, poultry meat is light in color and relatively bland in flavor, and is easily affected by the kinds of antimicrobial treatment that may be needed. Thus, it appears to be difficult to find a treatment that would be sufficiently effective, but without any adverse effect on the appearance, odor,

Table 20-5 Effect of Using Chlorinated Water Sprays in the Head Puller and Rinsing Hands between Each Carcass during Manual Cleaning of Neck Skins on the Transmission of a "Marker" Strain of *Escherichia coli*

Carcass no.	*Without Sprays in Head Puller*	*With Sprays in Head Puller*	*Without Hand Rinsing*	*With Hand Rinsing*
1	5.3[a]	5.7	6.5	6.2
10	2.7	2.5	4.4	2.5
50	2.1	0.9		
100	2.1	NF[b]	2.6	0.4
250	1.4	NF	1.3	NF
400			1.6	NF
500	0.9	NF	0.3	NF

[a] Log_{10} cfu g^{-1} of neck skin; Mean of three trials in each case.
[b] NF = not found.

Source: Reprinted with permission from G.C. Mead, W.R. Hudson, and M.H. Hinton, Use of a Marker Organism in Poultry Processing to Identify Sites of Cross-Contamination and Evaluate Possible Control Measures, *British Poultry Science*, Vol. 35, pp. 345–354, © 1994, Carfax Publishing Co.

flavor, or nutritional properties of the meat. Undesirable residues would also need to be avoided, as would any damage to the environment in disposing of waste treatment material. In addition, the proposed treatment must be acceptable to both consumers and legislators.

Despite the above difficulties, many attempts have been made to develop a suitable means of product decontamination. These have included a variety of physical and chemical methods, some of which were proposed for red meat, but may be also applicable to poultry.[24] Many of the treatments were purely experimental and have not been tested under commercial conditions. The physical methods include use of steam or hot water, high-intensity visible light, microwaves, high voltage pulsed electric fields, oscillating magnetic field pulses, high pressure, ultrasonic energy, and negative air ions. Physical methods have the advantage of avoiding any chemical residues but, to be effective against microbial contaminants, some of them could cause undesirable changes in the meat. A case in point is the application of steam, but cooking of the meat can be avoided by repeated exposure of the meat for very short periods of time. In one approach,[87] meat samples were subjected to superheated steam for 52 milliseconds or more in repeating cycles. The meat was then cooled rapidly by re-evaporating the condensate on the meat surface back into vacuum. Following inoculation, a reduction in listerias of almost 4 $\log_{10}$ units could be achieved with this method, without any visible effect on the meat. The steam treatment was carried out in a model system and was not ready for commercial use at that time.

Other proposed treatments include various chemicals, antibiotics, bacteriocins, and bioactive peptides. Chemical treatments (Exhibit 20-1) are relatively easy to apply while carcasses remain on-line and utilize either immersion or spraying processes. At present, the main contenders appear to be lactic acid and trisodium phosphate, both of which are acceptable for food use in the US and other countries, but have yet to be fully approved in the EU for treating poultry carcasses. The effectiveness of organic acids in reducing meat contamination is known to depend on the method of application, the concentration used, the time and temperature of treatment, and the extent to which microbial contaminants have become attached to the meat surface prior to treatment.[128] When carried out under optimal conditions, decontamination can be expected to reduce or even eliminate low numbers of foodborne pathogens. The process should be regarded as an adjunct to good processing hygiene rather than a substitute for it. In a comparison of nine different decontamination treatments involving a 20-second period of immersion,[1] trisodium or tripotassium phosphate (10%) were the most effective, reducing skin contamination by 10- to a 100-fold. However, the

Exhibit 20-1 Some Chemical Treatments Used Either Commercially or in Laboratory Trials for Microbial Decontamination of Processed Poultry Carcasses

Chlorine related	***Organic acids***	***Miscellaneous***
Chlorine gas	Acetic	ß-propiolactone
Hypochlorite	Lactic	Glutaraldehyde
Chlorine dioxide	Citric	Hydrogen peroxide
Salmide (a sodium chlorite-based oxy-halogen disinfectant)	Succinic	Hydrochloric acid
	Fumaric	Phosphoric acid
	Adipic	Lactoperoxidase system
	Malonic	Trisodium phosphate
		Lysozyme
		EDTA
		Ozone
		Polyphosphates
		Hydroxides
		Bacteriocins
		Iodophors
		Ethanol

Source: Adapted with permission from B.W. Sheldon, New and Novel Chemicals and Biological Approaches for Inhibiting Pathogens and Spoilage Microorganisms Associated with Food Systems, *Turkeys,* Vol. 44, No. 2, pp. 9–12, © 1996, Fancy Fowl Publications Ltd.

mechanism of the effect remains uncertain. Likely factors include the high pH (*ca.* pH 12.0), the detergent action, and detachment of microbial contaminants from the carcass surface.

The treatment that would be most effective in destroying foodborne pathogens on poultry meat is the use of ionizing radiation. The process is regarded as entirely safe as far as the product is concerned and it is not associated with any toxicological hazards or nutritional or microbiological problems.[147] At dose levels considered suitable for poultry (up to 3.0 kGy for chilled products), it has minimal effects on the sensory properties of the meat. Higher doses can be used for frozen poultry, depending on the temperature at which the product is treated. For whole carcasses, the high penetrative capability of gamma rays would appear to be needed but, for cut portions, it would be adequate to use the less penetrating electrons produced by linear accelerators. A particular advantage of irradiation is that it can be applied to products that are already packaged, thereby avoiding any possibility of post-treatment contamination. Some evidence suggests that the treatment dose for fresh chicken and turkey should not exceed 2.5 and 1.5 kGy, respectively, to avoid flavor changes in the cooked meat (Table 20-6). Such changes can only be avoided by treating the product in the frozen state. At 2.5 kGy, salmonellas would be virtually eliminated because the organisms are generally present on carcass meat in only low numbers, 90% of carcasses carrying <100 cells 100 g^{-1} of skin.[93] With irradiated, frozen carcasses, there is a further lethal effect of frozen storage.[91]

Irradiation may also double the shelf life of chilled poultry and *Pseudomonas* spp. the main spoilage organisms of meat stored aerobically under chill conditions are readily killed by the treatment. At the low-dose levels being recommended, however, a small proportion of the *Pseudomonas* population will usually survive and ultimately multiply to cause spoilage in the normal manner. It is rare to find a different spoilage microflora, although off-odors resulting from growth of organisms that are now classified as *Acinetobacter/Psychrobacter* spp. have been reported.[56]

Despite the considerable advantages of irradiation with regard to product safety and shelf life, the logistics of introducing the process would require careful consideration. Installing a facility for gamma irradiation would cost several million pounds (UK) or dollars (US); it may be necessary to establish regional treatment centers that could be shared, rather than plants belonging to individual companies. This approach has the disadvantage of incurring additional transportation costs. There is also the question of whether sufficient cobalt 60 would be available to satisfy the demand or suitable electron accelerators could be developed for whole carcasses. Regardless of feasibility, there is still the barrier of consumer resistance to food irradiation in many of the developed countries. Frequently, it is found that consumers do not understand the technology and are afraid that treated food will be hazardous, less nutritious, and of lower sensory quality.[62] In the US, consumers appear to take a more positive stance and, in one survey, about half of those questioned stated that they would be willing to purchase irradiated foods.[17] This contrasts with the situation in the UK, where neither the public nor the food industry appears to show any real interest in the process, and considerable effort would be required to inform the public of the specific advantages of irradiation, while allaying those fears that are based on common misconceptions.

Table 20-6 Doses of Ionizing Radiation for Different Kinds of Meat, Above Which Flavor Changes in the Cooked Meat Were Detectable by a Taste Panel

Type of Meat	*Dose (kGy)*
Turkey	1.50
Pork	1.75
Beef	2.50
Chicken	2.50
Lamb	6.25

Note: Samples in gas-impermeable pouches were irradiated at 4.4° to 10°C.
Source: Reprinted with permission from S. Sudarmadji and W.M. Urbain, Flavor Sensitivity of Selected Animal Protein Foods to Gamma Radiation, *Journal of Food Science*, Vol. 37, pp. 671–672, © 1972, Institute of Food Technologists.

20.4 RAW POULTRY PRODUCTS

Raw poultry meat is available in various forms, whether fresh or frozen, and includes whole carcasses and a variety of cut portions, with or without skin, to suit consumer demand for convenience items. Carcasses may also be self-basting or with added salt, polyphosphate, or herbs and spices, etc. Some, but not all carcasses, may include giblets and these are sometimes available separately, especially liver. In some countries, there is a demand for uneviscerated carcasses, including those that are hung like game birds, to enhance meat flavor. Cut portions include breast meat and thighs (with or without bone), drumsticks, and wings. After por-

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tioning in the processing plant is complete, fragments of meat remain on the residual bones and are harvested by machine. The mechanically separated meat (MSM) is in a finely comminuted state, but is one of the most important by-products and is used in soups, meatballs, patties, and various emulsion-type products, such as salamis and frankfurters.

As with other flesh foods, the bacteria that multiply most rapidly on chilled poultry meat are pigmented and nonpigmented strains of *Pseudomonas* spp., including *P. fluorescens, P. fragi,* and *P. putida.* These are accompanied by lower numbers of *Acinetobacter* spp., *Psychrobacter immobilis,* and an organism now recognized as a nonmotile form of *P. fragi.*[26] All these bacteria grow well on cut muscle surfaces, where moisture and nutrients are abundant, and some of the pseudomonads produce characteristic off-odors when populations reach *ca.* 10^8 cfu g^{-1}. Because whole carcasses first develop off-odors from the neck skin, some producers remove as much of this tissue as possible to gain a further day of shelf life. The mechanism of bacterial spoilage has not been determined for poultry meat, but is assumed to be the same as that of red meat. Thus, the spoilage pseudomonads are likely to grow preferentially on muscle glucose and, when this is exhausted, they will ultimately utilize amino acids, some of which can give rise to malodorous, volatile compounds that are associated with typical spoilage.

Much of the poultry meat sold in supermarkets is prewrapped, the immediate objective being to prevent drying of the surface and hence loss of weight, as well as to avoid damage to the product and microbial cross-contamination. In the case of air-chilled carcasses, the surface is relatively dry after chilling, with a reported water activity (a_w) of 0.970, while carcasses chilled by water immersion were found to have an a_w of 0.996.[114] After packaging, however, there was no significant difference in shelf life because, in the drier carcasses, moisture migrated to the surface from the deeper tissues.

Under chill conditions, storage temperature is the principal factor that determines shelf life by virtue of its effect on the growth of spoilage bacteria. The shelf life of whole carcasses packaged in oxygen-permeable film is usually 12 to 14 days at 1°C. With poultry sold unwrapped in a butcher's shop, for example, the drying of the surface may help to prolong shelf life, provided that chill storage is maintained. However, whole carcasses are unlikely to dry out uniformly and moist areas tend to remain. The most effective means of extending shelf life under aerobic conditions is to keep the product as cold as possible without causing it to freeze, ie, *ca.* –1°C. The effect of temperature on the shelf life of air-chilled eviscerated turkeys is shown in Figure 20-2.

Shelf life is also affected by the nature of the packaging film, which can modify the composition of the gaseous environment within the pack. All films will have some effect in this respect, and those that have low permeability to oxygen and carbon dioxide can significantly extend shelf life. In vacuum packs, with low-permeability film, oxygen is consumed during chill storage, while carbon dioxide accumulates due to residual tissue respiration and microbial metabolism. The increase in carbon dioxide inhibits growth of aerobic spoilage bacteria[25,43] and extends shelf life by this means. Even atmospheres containing only 3% carbon dioxide have been found to delay growth of pseudomonads on red meat,[35] although, at any concentration, the inhibitory effect depends upon maintenance of chill conditions. The increased level of carbon dioxide allows slower growing microorganisms that are unaffected by the prevailing conditions to become predominant. Eventually, these organisms will spoil the product, with off-odors that are less objectionable than those produced under aerobic conditions. The causative organisms include *Carnobacterium* spp. and other lactic acid bacteria and, less frequently, *Brochothrix thermosphacta,*

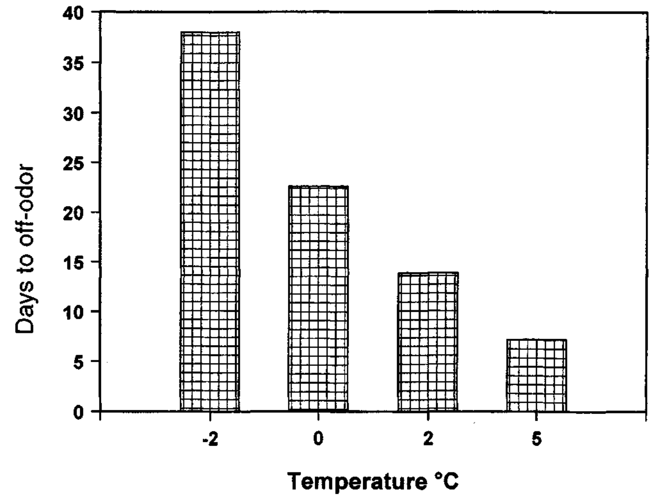

Figure 20-2 Effect of Storage Temperature on the Spoilage Rate of Air-Chilled, Eviscerated Turkeys. (Means of 10 carcasses in each case.) The carcasses were stored in oxygen-permeable polyethylene bags. *Source:* Data from E.M. Barnes, et al., The Effect of Storage Temperature on the Shelf-Life of Eviscerated Air-Chilled Turkeys, *British Poultry Science,* Vol. 19, pp. 77–84, © 1978, Carfax Publishing Co.

Shewanella putrefaciens, psychrotrophic coliforms, and *Aeromonas* spp.

There is only limited use of vacuum packaging for raw poultry meat and other systems of modified atmosphere storage are more common. One such system involves bulk packaging some 30 kg of product in an oxygen-impermeable bag, which is then evacuated and back-flushed with 100% carbon dioxide before being sealed. The pack can be delivered to retail stores and the unwrapped carcasses removed and displayed individually. Within the pack, a shelf life of 18 to 21 days at -2 to +1°C can be expected.[139] Gas-tight storage cabinets are sometimes used for the same purpose. In this case, each product is wrapped in a film that allows inward transmission of carbon dioxide. The benefits of storing poultry meat in atmospheres enriched with carbon dioxide have been known for many years and followed from the classical work of Ogilvey and Ayres.[103] Cut portions of chicken were stored either in air or air containing different concentrations of carbon dioxide. Within the range 0% to 25% carbon dioxide and at a storage temperature of 4.4°C, the ratio of shelf life in carbon dioxide to that in air ("storage index") was found to be a linear function of carbon dioxide concentration. The carbon dioxide affected both the lag phase and doubling time of the bacteria present.

Retailing of red meat in modified atmosphere packs (MAP) involves gas mixtures containing a high concentration of oxygen to maintain and enhance the red color of the meat due to formation of oxymyoglobin. Inclusion of oxygen is unnecessary for poultry meat, which is naturally much lighter in color, and most packs of poultry meat sold in this form do not include added oxygen.[22] However, 100% carbon dioxide would be unsuitable for retail packs because, at this concentration, some of the gas would dissolve in the tissues, causing the pack to collapse. Concentrations of 20% or more in nitrogen, for example, are usually employed.

Some researchers prefer to use mixtures of carbon dioxide and air, and this may be necessary for duck meat to safeguard the appearance of the product.[79] In a study of chicken breast and leg portions held at 2°C in atmospheres containing 0% to 80% carbon dioxide in air, aerobic plate counts were 10,000-fold higher after 14 days for portions held in air than those stored under 80% carbon dioxide.[50] The carbon dioxide-stored product was rated more highly for raw meat odor and overall acceptability by a sensory panel, and eating quality of the cooked meat remained acceptable for up to 35 days. An interesting feature of portions held in 80% carbon dioxide was a residual inhibitory effect on microbial growth when the product was transferred to aerobic conditions. This was not observed with lower concentrations of carbon dioxide. The change of gaseous environment may have extended the lag phase of the spoilage bacteria or the effect may have been due to the slow release of carbon dioxide dissolved in the tissues.

Only recently have large amounts of raw chicken been sold at retail in MAP because, previously, costs were too high. This type of presentation has been used more often for turkey and duck, which command a higher price. Specific gas mixtures are required for these meats. With duck portions stored at 1°C in 20% or 80% carbon dioxide in nitrogen, an unacceptable waxen or milky appearance has been observed in the skin.[80] The same effect occurred in pure nitrogen, but not in 20% carbon dioxide in air. The latter gas mixture extended shelf life from 14 to 21 days on the basis of raw meat odor and cooked meat flavor. Turkey, on the other hand, is particularly susceptible to oxidative changes during chill storage, and breast fillets were best packaged without air.[79] Fillets held at 1°C in 30% carbon dioxide in nitrogen showed no significant deleterious changes after 21 days.

20.4.1 Possible Hazards from Psychrotrophic Pathogens

Extending the shelf life of raw poultry by use of MAP raises the question of whether psychrotrophic pathogens that are always outgrown by spoilage pseudomonads under the usual aerobic conditions might multiply and reach hazardous levels when the pseudomonads and other aerobic spoilage bacteria are inhibited. The pathogens of concern include those strains of *Clostridium botulinum* that can grow and produce toxin down to 3.3°C, as well as *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Aeromonas* spp. In relation to botulism, vacuum and gas packaged foods have a remarkable safety record, with only seven outbreaks occurring worldwide between 1960 and 1991.[116] Most of these involved smoked fish products and resulted from gross temperature abuse. Fresh meats in MAP, including poultry, would present a relatively low risk of causing botulism because of the normal conditions of chill storage, the presence of a competing psychrotrophic microflora, and a defined shelf life. Also, the ultimate safeguard is that poultry meat always will be cooked before consumption. A potentially different situation arises with some further-processed, ready-to-eat poultry products, which will be discussed in a later section.

Studies on the growth of other psychrotrophic pathogens on raw meats have been reviewed,[34] revealing some conflicting results in the literature. In general, however, the ability of the organisms to multiply will depend on conditions of temperature, meat pH, and competing microflora and MAP conditions do not appear to involve any additional hazard. At carbon dioxide concentrations above 75%, listerias, yersinias, and aeromonads tend to be inhibited on meats, while the higher the carbon dioxide concentration and the lower the pH and storage temperature, the greater is the growth inhibition of these organisms. Thus, holding chilled poultry under 100% carbon dioxide can be expected to prevent or retard any multiplication of vegetative, psychrotrophic pathogens. Even growth of psychrotrophic *C. botulinum* is likely to be delayed under these conditions.[30]

20.4.2 Uneviscerated Carcasses

In some countries, there is still a significant trade in chicken carcasses that have been plucked, but not eviscerated, by the producer. Subsequently, the carcass may be eviscerated by a butcher or in the kitchen of the consumer. Another, similar product is a special breed of turkey that is produced in the UK for the Christmas market. In this case, however, the carcasses are hung by the processor at 4°C for up to 2 weeks to allow flavor development and then eviscerated before distribution. Uneviscerated poultry, with head and feet still intact, are sometimes referred to as "New York dressed" (NYD), while carcasses from which only the intestines have been removed through a small opening are termed "effilé." The more traditional NYD carcass is less prone to spoilage than its eviscerated counterpart, as long as the carcass is kept cool and dry, with an intact skin. These conditions provide little opportunity for multiplication of the usual aerobic spoilage bacteria, and small processors can begin production well in advance of the Christmas season to meet demand. It has been shown[8] that NYD carcasses were still acceptable after 18 days at 4°C, while those that had been eviscerated initially were spoiled after little more than a week (Table 20-7). At lower temperatures, NYD carcasses could be expected to keep even longer.[6]

Table 20-7 Shelf Life of Eviscerated and Uneviscerated, Air-Chilled Chicken Carcasses Stored at 4°C. Mean and range indicated by ( ) of 10 carcasses in each case.[8]

	Time (days) to	
Type of Product	*Slight Off-Odor*	*Off-Odor*
Eviscerated, wrapped	5.6 (5–6)	7.6 (6–8)
Eviscerated, not wrapped	7.9 (5–11)	9.8 (7–13)
Uneviscerated, not wrapped	>18.0[a]	>18.0[a]

[a] Neither greening nor off-odors. Wrapped carcasses were held in food-grade "polythene."

Source: Data from E.M. Barnes and C.S. Impey, The Shelf-Life of Uneviscerated and Eviscerated Chicken Carcasses Stored at 10°C and 4°C, *British Poultry Science*, Vol. 16, pp. 319–326, © 1975, Carfax Publishing Co.

Spoilage of NYD poultry can be due to one of three possible causes. If the dryness of the surface is not maintained, aerobic spoilage bacteria will multiply and produce off-odors in the usual manner. Alternatively, carcasses kept uneviscerated for prolonged periods can develop a meat flavor that some individuals would find unacceptable, ie, a "gamey" or "gutty" flavor. The third, more common type of spoilage is due to microbial production of hydrogen sulfide in the intestines and diffusion into the musculature. At the surface of the carcass, in the presence of air, the hydrogen sulfide reacts with heme pigments of blood and muscle to form sulfur derivatives.[6] This leads to an unacceptable greening of the skin, a condition that is sometimes termed "greenstruck." The greening, which develops at a rate dependent on storage temperature, is first evident in the region of the large intestine, where the concentration of sulfide-producing bacteria is greatest. From the vent area, greening spreads upward, while the muscle tissue remains relatively free from microorganisms. The potential for greening is reduced by starving birds prior to slaughter, thus minimizing microbial growth in the intestine. It is generally thought that eviscerating carcasses outside the processing plant is likely to increase the risk of cross-contaminating other foods with fecal bacteria, and could be a hazard for foods that are ready to eat. Also, NYD carcasses cannot be properly examined by the inspectorate at the time of production and, theoretically, unfit carcasses could pass into the human food chain. These problems do not arise with carcasses that are kept in chill storage by the processor and ultimately eviscerated on the same premises.

20.5 FURTHER-PROCESSED PRODUCTS

Further processing converts carcass meat into a variety of value-added products that are popular convenience foods. It involves operations such as portioning, deboning, size reduction, tumbling, massaging, reforming, emulsifying, breading, battering, and marinading. The products may be cooked, cured, smoked, dehydrated, or canned, or appropriate combinations of these processes may be used.

Cutting of meat and the various steps involved in product manufacture increase the opportunities for microbial contamination, and it has been reported that counts can increase up to six-fold during plant cutting operations.[68] More centralized cutting and packaging of poultry, as occurs now in processing plants, has improved the control of microorganisms.[33,48] Ingredients of poultry products can also increase microbial contamination, especially spices, which sometimes contain large numbers of bacterial spores. In relation to manufactured products, use is made of the highly comminuted mechanically separated meat (MSM), which may serve as an ingredient or as a binder in reformed products. The process used to obtain MSM causes considerable cellular destruction, which releases nutrients and makes this material more prone to microbial growth and spoilage, as well as oxidative rancidity. Contamination of MSM is influenced by the type of raw material used, eg, whether or not it includes skin, which is more highly contaminated than muscle. Aerobic plate counts of 10^4–10^5 g^{-1} can be achieved, but counts are sometimes higher. MSM is normally held at 0° to 2°C for up to 48 hours or frozen before use.

Products that are sold as "ready to eat" receive an appropriate heat treatment that destroys most vegetative bacteria. The methods employed include water-bath pasteurization, boiling, baking, barbecuing, and frying. In some cases, eg, baked, barbecued, and fried items, the product is packaged after heat processing and therefore is susceptible to post-process contamination. In the UK, coated, flash-fried products that may appear to be cooked are, in fact, virtually raw and have caused food poisoning because of inadequate cooking by consumers.[119] This type of product needs clear labeling to indicate the further heating requirements.

Earlier studies on the safety of cooked poultry products were largely concerned with the control of *Salmonella* and *Staphylococcus aureus*, and it was shown that cooking to an internal temperature of 71.1°C eliminated any significant risk of human food poisoning from these organisms.[151] In inoculation studies on turkey roll,[20] the component pieces of meat were inoculated by dipping in a broth culture of the relatively heat-resistant *S. senftenberg* 775W to give a concentration of >106 cfu g^{-1} and pressed together to form rolls in a polyethylene casing. After cooking in an oven to an internal temperature of 65.6°C or heating at 62.8°C for 4 hours, the number of salmonellas was reduced to <0.3 g^{-1} or cm^{-2}. The heat treatment used to inactivate salmonellas would not be sufficient to destroy clostridial spores, including those of *Clostridium perfringens*. The heating process drives off oxygen, lowers the E_h of the meat, and provides a heat shock that can promote spore germination. Subsequent outgrowth must be controlled by prompt and efficient chilling of the cooked product. However, the chilling process may not prevent the growth of any psychrotrophic strains of *Clostridium botulinum*, especially in uncured and possibly smoked poultry products held in MAP, with a shelf life greater than 10 days. Manufacturers need to take account of this potential hazard in setting cooking temperatures and "use by" dates. To obtain a 6-$\log_{10}$ reduction in spores of psychrotrophic *C. botulinum*, a treatment equivalent to heating at 90°C for 10 minutes has been recommended.[116]

In relation to the risk of recontaminating cooked products prior to packaging, a study on foil-wrapped oven-roasted turkey rolls showed that post-cooking processing at two US factories had no significant effects on aerobic plate counts, but 8 of 28 rolls examined after packaging contained coagulase-positive staphylococci.[84] The organisms could have been acquired by cross-contamination from raw meat, contaminated equipment, or the hands of operatives. Under present conditions, where higher standards of handling hygiene are widely practiced, this problem should be avoidable.

Listeria monocytogenes is a common contaminant of raw poultry meat and may also be found in the further processing environment, where it is often associated with floors, drains, cleaning aids, processing equipment, and food contact surfaces. Heating foods to an internal temperature of 70°C for 2 minutes is recommended to ensure destruction of *L. monocytogenes*.[36,67] Occasional reports of unusually high heat resistance have been attributed to uneven heating of the product or the protective effects of fat or drying. Because human listeriosis can be a serious disease, with a significant level of mortality, contamination of ready-to-eat foods with *L. monocytogenes* is potentially hazardous and may lead to the recall of affected products from retail stores. Recalled products have included turkey frankfurters, chicken spread, and chicken salad.[32] The widespread occurrence of *L. monocytogenes* is a problem in relation to product contamination, even with rigorous application of HACCP principles, and low levels of contamination are likely to be present, ie, up to 100 g^{-1}, in a proportion of samples examined.[120] The organism survives well on poultry and grows better than on other kinds of meat, especially above pH 6.0.[38] It is also relatively resistant to curing agents, but may be adversely affected by some smoking treatments.[86]

Given the great diversity of further-processed poultry products, it is not surprising that there is no systematic information on the spoilage characteristics of these items during chill storage. In practice, the shelf life of cooked products is related to the severity of the cooking process, the extent of any post-processing contamination, the type of packaging used, and the time and temperature of storage. For unwrapped carcasses cooked in a circulating, moist-air oven at 85°C, with the meat temperature maintained for at least 50 minutes, only spore formers were detected, mainly *C. bifermentans* and *Bacillus subtilis*.[107] Storage at an abuse temperature of 16°C increased the numbers of vegetative organisms and off-odors were evident after 10 days. At 1° to 3°C, the carcasses showed no sign of spoilage after 4 weeks, and vegetative organisms did not increase in number.

Vacuum packaged, cured, and smoked chicken carcasses had a shelf life of 84 days at 1° to 5°C, following cooking to 70°C or 80°C, and 104 days at 1°C, when cooked to 99°C.[89] These conditions severely limited the growth of yeasts and molds, which otherwise would have spoiled the product within 8 days at 1°C. Another study, involving smoked chicken halves, showed that molds caused visible spoilage at 25°C and 15°C.[102] At these temperatures, spoilage was evident after 9 and 12 days, respectively.

Cooking usually destroys the psychrotrophic bacteria that cause spoilage of raw meat stored aerobically under chill conditions, although recontamination of the product may occur post-cooking. It has been reported that pseudomonads can spoil creamed or gravy products,[110] whereas, in products that are salted, as in kosher processing, the predominant spoilage organisms are salt-tolerant "*Moraxella*" spp.[59]

20.6 CONCLUDING REMARKS

In the developed world, poultry meat production is a highly intensive and efficient industry, which continues to expand in response to consumer demand. Despite its popularity, however, poultry meat is frequently contaminated with foodborne pathogens, such as *Salmonella* and *Campylobacter* spp., organisms that, in many countries, are major causes of human enteritis. While the ultimate safeguard is adequate cooking of the meat and good handling hygiene in the kitchen, the risk of illness occurring is inevitably influenced by flock infection and its control in the hatchery and on the farm, and by the use of hygienic practices in the processing plant. The transmission of foodborne pathogens in poultry production is significantly affected by the intensive nature of present systems for breeding, hatching, growing, and processing the birds. At present, there are no acceptable means of eliminating all foodborne pathogens from raw poultry, and control measures, if they are to be effective, must be applied uniformly at all stages in the production chain in order to minimize product contamination.

Although the situation on farms tends to favor the spread of any pathogens present, because large numbers of birds are reared together in close proximity, the controlled environmental conditions that are necessary for optimal poultry production can also be exploited to prevent flock infection. In this respect, the measures necessary to achieve high standards of biosecurity are well known, and control of *Salmonella*, at least, can be reinforced by the use of competitive exclusion treatment, vaccination, acid treatment of feed, etc, as appropriate. These control measures, either singly, or in combination, are usually applied at the upper end of the production pyramid, but much less so in relation to broiler flocks. The main reason for this is cost. In what is, essentially, a high volume, low profit margin industry, there are currently no financial rewards for producing meat that has only minimal levels of pathogens or, theoretically, is pathogen-free. Customers expect that the foods they purchase will be free from any hazard to human health, and they appear unwilling to pay any premium for foods produced in such a way that the risks are negligible.

At the processing plant, continuing developments in machinery and equipment have facilitated increased rates of carcass production, while maintaining product yield. Over the years, however, the process has shown little improvement in relation to hygiene control and, when improvements in individual items of equipment have been made, their use within the industry has been limited. Nevertheless, the importance of hygiene control in the processing plant is widely recognized because it ensures that the final product is of acceptable microbiological quality, with low numbers of any foodborne pathogens or spoilage organisms. The main problem with foodborne pathogens in processing is the considerable opportunity for cross-contamination, which cannot be prevented by the current GMP and HACCP programs alone. There is clearly a need to introduce a terminal decontamination treatment for carcasses that would provide a much-needed CCP and this is being explored. At present, it seems unlikely that the use of ionizing radiation would be widely accepted for the purpose and, in any case, there could be some logistical problems in treating the very large amounts of poultry meat being produced. In practice, any decontamination process must be effective, safe for both plant personnel and consumers, and not damaging to the environment if disposal of treatment material is involved. Suitable treatments are becoming available, but will need to be sanctioned more widely by regulatory bodies and their use encouraged.

Poultry meat is highly suitable as a raw material for product development and, with its light color and delicate flavor, can be transformed into a wide range of value-added convenience foods. With ready-to-eat products, there have been concerns over the possible presence and outgrowth of spores of psychrotrophic strains of *C. botulinum* and low rates of contamination with *L. monocytogenes*, which are currently unavoidable. Despite these problems, further-processed poultry products will continue to be developed and make a significant contribution to the high level of poultry meat consumption worldwide.

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21 Fresh and Processed Fish and Shellfish

Lone Gram and Hans Henrik Huss

CHAPTER CONTENTS

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21.1 INTRODUCTION

Fish and shellfish constitute a very important part of the diet in many parts of the world and with the increasing world population, effective utilization of all food resources is of major importance. While "milk," "meat," and "egg" may each denote a relatively well-defined group of raw material, "fish" covers an enormous range of species; currently approximately 20,000 different species have been identified. These are caught or harvested over a wide range of conditions, from the cold waters of Greenland to the warm waters of Southeast Asia, from freshwater lakes to oceans with 3% to 4% salt. In terms of composition of the raw material, fish range from white fleshed low-fat fish with large regular fillets to small, fatty pelagic fish species with many bones in the fillet. As opposed to many other food raw materials, fish are mainly harvested from a wild population and only a small, but increasing, fraction is cultured in mari- or aquaculture. Despite these variations, there are many general features in the microbiology of the fish and fish products.

Fish and shellfish are processed using almost every known food preservation technique; however, the trend in most parts of the world is toward more fresh and less preserved products (Table 21-1).

Most studies of the microbiology of fish and fish products have been carried out using a few selected fish species, like cod and plaice, from temperate, marine waters. During the last five years the total production of fish and shellfish has been just under 100 million tonnes.[77,79] In the same period, the so-called developing countries have surpassed the developed countries in terms of amount of fish supplied, and approximately 60% now comes from these countries.[77] This emphasizes the need for an understanding of the microbiology of fish caught or harvested in warmer, tropical waters, and utilized as raw materials for further processing.

In contrast to the decline in wild fish catches, the amount of reared fish and shellfish increased from 7 million tonnes in 1984 to 13.9 million tonnes in 1992.[48] While the amount of wild fish that can be used for human consumption is expected to remain at 60 million tonnes per year, it is estimated that the amount of reared fish will increase to 25 million tonnes in year 2000.[48] Farming of fish has obvious advantages in terms of ability to control chemical composition of the fillet, growth rate, harvesting time, etc. In microbiological terms, farming of fish poses a special problem partly resulting from the usage of antibiotics which, as in any intensive farming, may cause development of antibiotic-resistant bacteria that, via the food, can be transferred to the consumer.

Table 21-1 Major Groups of Fish Products

	Percentage of the Catch Used for Human Consumption					
Year	*Total*	*Fresh*	*Frozen*	*Cured*	*Canned*	*Other, Including Animal Feed*
1982	71.1	19.4	25.3	12.8	13.6	28.9
1992	72.8	27.0	24.1	9.3	12.4	27.2

Source: Data from FAO, State of Food and Agriculture, *FAO Agriculture Series 26,* U.N. Food and Agriculture Organization, and H.H. Huss, Assurance of Seafood Quality, *FAO Fish. Technical Paper 334,* U.N. Food and Agriculture Organization.

The growth and metabolism of microorganisms give rise to two hazards in food (fish) products, disease (infection, intoxication), and/or spoilage.

Statistics of foodborne diseases are generally quite inaccurate as only a fraction of diseases are reported and only in some (of the reported) cases are the etiological agents identified. Statistics from the United States (US), Canada, and the Netherlands show that seafood is implicated in 8% to 10% of the outbreaks of foodborne disease[121] and in some areas, seafood products are the most commonly implicated vehicles.[262] Thus, in Korea and Japan fish and other seafood products were the most commonly implicated vehicles of foodborne disease accounting for 32% and 22%, respectively, of the outbreaks.[163] However, in several of the reported outbreaks only few people were affected,[30] and seafood only accounted for 3.6% of the cases of foodborne illness in the US from 1978 to 1987.[174] In contrast, the largest foodborne disease outbreak ever recorded was caused by hepatitis A in China where 290,000 people were infected by undercooked shellfish.[258] The causes of disease vary depending on the raw material. Marine toxins (eg, ciguatera) and scombroid toxin are the most important agents of disease transmitted by fresh and frozen fish[121] (see Chapter 54) whereas viruses and marine vibrios are implicated in 38% of diseases transmitted by shellfish.[121,174,223] The etiological agents responsible for health hazards of seafoods are listed in Tables 21-2 and 21-3. Bacteria indigenous to the aquatic environment are usually present on the raw material, although mostly in low numbers. Apart from some vibrios that may be found in high numbers (see below), prevention of disease caused by indigenous organisms relies on prevention of bacterial growth. Nonindigenous bacteria are either part of the general environment (eg, *Listeria monocytogenes* and *Clostridium botulinum* type A and B) and as such often present in water, or are found on seafood as a result of human (often fecal) contamination. For a thorough discussion of all the agents, including parasites, and their importance in fish products, the reader is referred to Huss[121] and Chapters 38 through 54 in Vol. II of this book.

It has been estimated that 25% of all fresh food spoil because of microbial action,[82] and the Food and Agricultural Organization (FAO) estimates that post-harvest losses in the fishery sector are in the same range. While these losses, particularly in tropical countries, are to some extent due to insect infestation, microbial spoilage is of major importance.

21.2 CHEMICAL COMPOSITION OF FISH AND SHELLFISH

The chemical composition of fish varies depending on the species, age, environment, sexual cycle,

Table 21-2 Bacterial Health Hazards Associated with Fish and Shellfish Products

Indigenous		*Nonindigenous*	
Toxin Preformed in Product	*Infection*	*Toxin Preformed in Product*	*Infection*
Clostridium botulinum nonproteolytic type B, E, and F psychrotolerant histamine-producing bacteria (photobacteria)	*Listeria monocytogenes*[a] *Vibrio cholerae* *Vibrio parahaemolyticus* *Vibrio vulnificus* *Aeromonas hydrophila* *Plesiomonas shigelloides* *Shewanella alga*[b]	*Staphylococcus aureus* *Clostridium botulinum* proteolytic type A and B[c] mesophilic histamine-producing bacteria (*Morganella morganii*)	*Salmonella* spp. *Shigella* spp. *Escherichia coli*

[a] *L. monocytogenes* seems to persist in the aquatic environment once introduced

[b] *S. alga* is a recently defined species that has not been implicated in foodborne disease but has caused wound infection and bacteremia through contact with warm water

[c] *Clostridium botulinum* type A and B occur in the general environment and are as such frequently found in water.

Source: Data from H.H. Huss, Assurance of Seafood Quality, *FAO Fish. Technical Paper 334,* U.N. Food and Agriculture Organization.

Table 21-3 Health Hazards Caused by Viruses, Marine Toxins, and Parasites Associated with Fish and Shellfish Products

Viruses	*Marine Toxins*	*Parasites*[a]
Hepatitis A	Tetrodotoxin[b]	Nematodes
Norwalk	Ciguatera	*Anisakis simplex*
Calicivirus	Shellfish poisoning	*Gnathostoma* spp.
Astrovirus	Paralytic[b]	Cestodes
Non A–Non B	Diarrhetic	*Diphyllobothrium latum*
Snow mountain agent	Neurotoxic	
	Amnesic	Trematodes
		Clonorchis spp.

[a] See Huss[121] for a more exhaustive list.

[b] It has recently been speculated that bacteria are the producers of some marine toxins: tetrodotoxin[245] and paralytic shellfish toxin (see Chapter 54).

Source: Data from H.H. Huss, Assurance of Seafood Quality, *FAO Fish. Technical Paper 334,* U.N. Food and Agriculture Organization.

etc, but consists of approximately 20% protein, 1% to 2% nonprotein dry matter, the remaining 78% being a combination of water and lipid. The proportion of lipid and water varies dramatically depending on species and season. This rarely influences the microbiology of the product but affects quality and is of major technological importance due to problems caused by oxidation of the polyunsaturated fatty acids of fish lipids. A major difference from the mammalian muscle is the very low level of carbohydrate because fish do not, as do warm-blooded animals, accumulate glycogen. This has important consequences for the microbiology of fish as the pH *postmortem* is >6.0 in most fresh fish, which is higher than in mammalian muscle (Figure 21-1). Bivalve molluscan shellfish (clams, oysters) differ from the other aquatic animals because they are able to accumulate glycogen (0.5% to 6.8% depending on spawning cycle) in the muscle.[44]

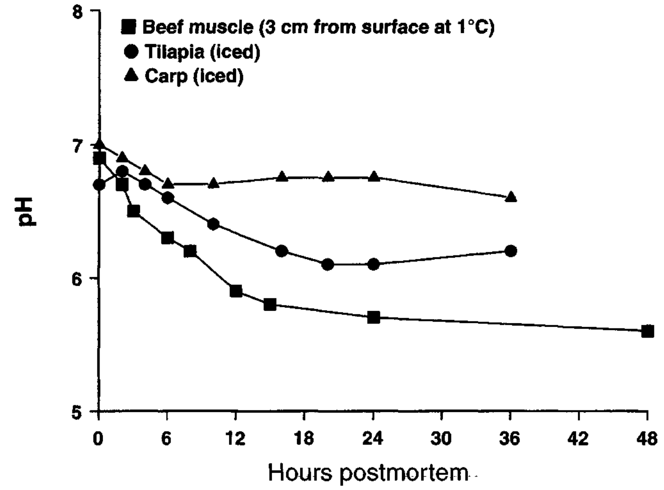

Figure 21-1 pH Changes *Postmortem* of Beef Muscle (*M. longissimus dorsi*), Tilapia (*Oreochromis aureus/niloticus* hybrid), and Carp (*Cyprinus carpio*). *Source:* Data from C.A. Curran et al., Cold Shock Reactions in Iced Tropical Fish, *Journal of Food Technology,* Vol. 21, pp. 289–299, © 1986, Institute of Food Technologists, and P.V. Tarrant and C. Mothersill, Glycolysis and Associated Changes in Beef Carcasses, *Journal of the Science of Food and Agriculture,* Vol. 28, pp. 739–749, © 1977.

The most important chemical fraction in relation to the microbiology of fish products is the nonprotein nitrogen (NPN) fraction. This consists of low molecular weight, water-soluble, nitrogen-containing compounds including amino acids, nucleotides, and trimethylamine oxide (Table 21-4). The NPN fraction is the major bacterial growth substrate. In a classical experiment, Lerke et al.[168] separated fish juice into a high molecular weight fraction (proteins) and low molecular weight fraction (NPN compounds) and showed that while spoilage bacteria grew well in the former, spoilage off-odors were only, and to the same extent as in fresh fish, produced in the NPN-containing fraction. Differences in the composition of the NPN fraction influence both potential bacterial health hazards and bacterial spoilage, as discussed below.

Trimethylamine oxide (TMAO) is one of the most important components of the fish muscle. It is an

Table 21-4 Nonprotein Nitrogen (NPN) Compounds of Fish and Crustacean Muscle

	mg 100 g^{-1} Wet Weight			
Compound	*Cod*	*Herring*	*Shark*	*Lobster*
Amino acids, total	75	300	100	3000
Arginine	<10	<10	<10	750
Glycine	20	20	20	100–1000
Glutamic acid	<10	<10	<10	270
Histidine	<1.0	86	<1.0	—
Proline	<1.0	<1.0	<1.0	750
Creatine	400	400	300	0
Betaine	0	0	150	100
Trimethylamine oxide	350	250	500–1000	100
Anserine	150	0	0	0
Carnosine	0	0	0	0
Urea	0	0	2000	—
NPN, total	1200	1200	3000	5500

Source: Data from J.M. Shewan, The Biodeterioration of Certain Proteinaceous Foodstuffs at Chill Temperatures, in *Industrial Aspects of Biochemistry,* B. Spencer, ed., pp. 475–490, © 1974, North Holland Publishing Co.

odorless compound typical of marine fish species,[108] particularly the gadoid fish, but contrary to earlier beliefs it also occurs in high concentrations in some freshwater fish species, eg, tilapia and Nile perch.[13,102,213] TMAO can be used by several bacteria as a terminal electron acceptor in anaerobic respiration. Reduced TMAO, trimethylamine (TMA), smells of old fish and is perhaps one of the most typical components of the odor profile of spoiling fish. For a number of fish species, TMA is used as a chemical indicator of spoilage.[203] The reduction potential of TMAO/TMA is +120 to +140 mV in nutrient broth at pH 7.0.[35] The redox potential in a fish fillet is positive (up to +100 to +300 mV probably depending on pH) as long as TMAO is present whereas E_h drops dramatically to negative values (to –300 mV) once TMAO is reduced.[124] The positive redox potential of fish may influence the microbiology, such as the growth and metabolism of anaerobic organisms. *C. botulinum* type E grows and produces toxin in fresh fish irrespective of the presence of TMAO.[128] Using O_2 to adjust Eh, it was shown that at 3.25% NaCl, the organism was much more sensitive to changes in Eh,[177] and the presence of TMAO has been suggested as an extra hurdle against growth of *C. botulinum* in salted fish.[129] Spoilage of sugar-salted herring by strict anaerobic organisms is only seen at low Eh, that is, if TMAO or any other oxidizing compound (like nitrate) is not present.[154]

Elasmobranches discharge nitrogen by accumulation of urea, which is a protein-denaturing agent. TMAO counteracts the protein-denaturing effect of urea,[275] and this is believed to be the reason for the high TMAO concentration of elasmobranches (Table 21-4). While TMAO is considered nontoxic to mammals, very high concentrations of TMA may, in fact, be toxic. Greenland sledge dogs that ingest large amounts of raw shark meat suffer from symptoms of TMA poisoning. It has been suggested that this is due to the reduction of TMAO by the dog intestinal microflora.[14]

The amino acid histidine is present in free form in herring and in scombroid fish species such as tuna and mackerel. Some bacteria, particularly among the Enterobacteriaceae, are able to decarboxylate this amino acid resulting in the formation of histamine. Histamine together with other biogenic amines (tyramine, spermine) may be implicated in a hypersensitive reaction in humans known as scombroid poisoning. Histamine formation is mainly a problem caused by temperature abuse of raw fish,[251] but has also been reported to take place in fish stored at low temperatures.[3,271] For further information, see below and Chapter 54.

The pool of free amino acids provides important substrates for bacterial growth.[222] The decomposition of the sulfur-containing amino acids cysteine and methionine is important in spoilage as it results in formation of hydrogen sulfide and methyl mercaptan, respectively.[109,110] The very high content of certain amino acids in crustaceans (Table 21-4) contributes to their typical, sweet flavor.

Some of the properties of fish muscle that influence the microbiology of fish and shellfish are summarized in Table 21-5.

21.3 MICROBIOLOGY OF FRESHLY CAUGHT FISH AND SHELLFISH

Microorganisms are found on all the outer surfaces (skin and gills) and in the intestines of live and newly caught fish. The number of culturable organisms varies with a normal range of 10^2–10^6 cfu cm^{-2} on the skin surface (Tables 21-8 and 21-9),[173] and the

Table 21-5 Properties of Muscle from Newly Caught and Spoiled Fish Compared with Those of Muscle from Fresh Beef

	Value/Characteristic		
Parameter	*Fresh Fish*	*Spoiled Fish*	*Fresh Beef*
Water activity	0.98	0.98	0.98
Redox potential	positive (+100 to > +300 mV)	negative (–200 to –400 mV)	negative
pH	high (6–7)[a]	6.5–7.5	low (5.4–5.5)
Nutrients	rich in nonprotein nitrogen free amino acids (75–3000 mg 100 g^{-1}) TMAO (0–350 mg 100 g^{-1}) nucleotides	—	rich in nonprotein nitrogen free amino acids (350 mg 100 g^{-1}) no TMAO
Structure	low in carbohydrate (<0.5%) skin/slime with some protective function	— —	high in carbohydrate (1% to 2%)

[a] Some fish species (halibut, tuna) may have a *postmortem* pH of approximately 5.5.

gills and the intestines both contain between 10^3 and 10^9 cfu g^{-1}.[239] As in other environments, not all the microorganisms may be culturable, thus Bernadsky and Rosenberg[25] found 4×10^8 bacteria cm^{-2} on cornetfish were stainable by the DNA stain DAPI (4,6-diamidino-2-phenylindole) or could be visualized by scanning electron microscopy but only 2×10^4 bacteria cm^{-2} were culturable on marine agar incubated at 25°C for 3 days.

The bacterial flora on newly caught fish depends on the environment in which it is caught rather than on the fish species.[241] Fish caught in very cold, clean waters carry lower numbers than fish caught in warm waters.

21.3.1 Fish and Shellfish from Temperate Waters

Wild Fish and Shellfish

The microflora on temperate water fish and shellfish is dominated by psychrotrophic or psychrophilic, Gram-negative, rod-shaped bacteria belonging to the genera *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Alkaligenes*, *Shewanella*, and *Flavobacterium*. Members of the *Vibrionaceae* (*Vibrio* spp. and *Photobacterium* spp.) and the *Aeromonadaceae* (*Aeromonas* spp.) are also common aquatic bacteria and typical of the fish flora (Table 21-6). *Aeromonas* spp. are typical of freshwater fish, whereas a number of bacteria require sodium for growth and are thus typical of marine waters. These include *Vibrio*, *Photobacterium*, and *Shewanella*. *Shewanella putrefaciens* is characterized as sodium requiring, but strains of *S. putrefaciens* can also be isolated from freshwater environments.[62,103,248] Gram-positive organisms such as *Bacillus*, *Micrococcus*, *Clostridium*, and *Corynebacterium* can also be found in varying proportions (Table 21-6). Lactic acid bacteria are seldom part of the dominant flora, but are present at levels of $10–10^3$ g^{-1}.[151,235]

Table 21-6 Bacterial Flora on Newly Caught Fish and Shellfish from Tropical and Temperate Marine and Fresh Waters

	Incidence (%)			
	Temperate		*Tropical*	
Species	*Marine*	*Fresh*	*Marine*	*Fresh*
Gram-negative				
Pseudomonas	0–70	0–22	0–53	0–16
Moraxella		0–14	0–52	0–36
Acinetobacter		0–11	0–15	0–8
"Achromobacter"[a]	5–50	0–10	0–15	0–19
Alcaligenes			0–10	0–10
Flavobacterium	2–25	0–6	0–54	0–13
Vibrio[b]	0–60		0–80	
Aeromonas		0–30		0–2
Enterobacteriaceae[c]		0–18	0–10	
Chromobacterium			0–20	
Gram-positive				
Micrococcus	0–53	0–10	0–60	0–30
Staphylococcus			0–41	0–18
Bacillus[d]	0–24		0–42	0–5
Coryneforms	0–10	0–12	0–55	0–5
Lactic acid bacteria			0–3	

[a] *Achromobacter* denotes Gram-negative, nonfermentative rods. Probably covers strains now identified as *Acinetobacter* and *Moraxella*. Includes also *Shewanella putrefaciens*, which is found in low numbers on newly caught fish.

[b] Includes also *Photobacterium phosphoreum*.

[c] In clean waters mainly psychrotrophic strains (eg, *Serratia liquefaciens* or *Hafnia alvei*).

[d] Clostridia may be isolated in low numbers.

Data from 6, 19, 31, 47, 75, 89, 103, 116, 157, 173, 216, 241, 253, 276.

Several types of *C. botulinum* are found in the aquatic environment. However, the nonproteolytic type E, which is psychrotrophic, is believed to be truly indigenous and occurs especially in temperate and subarctic zones.[119] *C. botulinum* is found in most sediments and on fish from different parts of the world. Quantitative data are scarce; however, numbers of spores in sediments are usually less than 100 g^{-1} although higher values have been reported in the Great Lakes in the US and in the Sound in Scandinavia.[63] It is hypothesized that the organism proliferates in dead fish and aquatic mammals[120] and the increasing environmental problems in the oceans, anoxic conditions, and mass fish mortality may have increased the numbers of *C. botulinum* in sediments and wild fish.

The proportions of the different bacterial groups vary (Table 21-6) and comparing the exact numbers is difficult, partly due to the change in classification of several of the organisms. The organisms that in earlier publications were identified as *Achromobacter* spp. include the following: (1) nonmotile rods, which are now either *Moraxella* or *Acinetobacter*; (2) motile rods now assigned to *Alcaligenes*; and (3) certain nonfluorescent rods now identified as *Pseudomonas* spp. or *Alteromonas* spp.[115]

Aeromonas spp. proliferate in the intestinal tract of freshwater fish[252] where they may occur in numbers of $10^5–10^7$ cfu g^{-1} and up to 10^7 cfu g^{-1} of vibrio-like organisms can be present in the intestinal tract of cod[161] and turbot.[274] *Photobacterium phosphoreum*, which can be isolated from the surface of fish, can also be isolated in high numbers ($10^7–10^8$ cfu g^{-1}) from the intestinal tract of some marine fish species.[192,269]

Farmed Fish and Shellfish

The natural bacterial flora on farmed fish and shellfish from temperate waters is similar to the microflora of wild fish.[248] However, as the microflora on fish is a reflection of the environment, fish reared in ponds or in shallow near-coast waters are closer to man (and his wastage) than are the wild fish, and this may cause a higher frequency of contamination with bacteria nonindigenous to water. Thus *Listeria monocytogenes* was not isolated from unpolluted ocean waters and spring water, but the organism could frequently be isolated from surface waters (up to 62% positive samples) and polluted seawater (up to 33% positive samples).[130] It is therefore not surprising that low levels of *L. monocytogenes* are found on raw fish, eg, salmon, and on products that do not receive a listericidal treatment, eg, cold-smoked salmon.[21,130] Although most investigators have presented qualitative data, Huang and Leung[117] reported low counts of *Listeria* spp. (2–20 g^{-1}) on caged channel catfish, and it is likely that in general, the numbers of *L. monocytogenes* on raw fish are very low. A few investigations state numbers on fish products like cold-smoked salmon and these are discussed in Section 21.5.

The highest ever recorded number of *C. botulinum* type E spores on fish (5.3 g^{-1}) was found in Danish farmed trout.[127] As the organism proliferates in dead fish and aquatic mammals, it was suggested that the use of wet fish as aquaculture feed, as well as poor hygienic measures, was the reason for this high number. During recent years, the use of wet feed has been prohibited.

As fish and shellfish become farmed animals, diseases become a major problem. The use of antibiotics, particularly of tetracyclines, has been very common, thus 50,000 kg of antibiotics were used in 1987 in Norway for the production of 4,000 tonnes of salmon. Fortunately, the development of vaccines have caused a decrease in the amount of antibiotics used, and in 1993 only 6,000 kg of antibiotics were used in Norway for the rearing of 170,000 tonnes of salmon.[11] A lot of effort is currently being put into development of vaccines and probiotic treatments; however, the usage of antibiotics has been, and continues to be, very widespread. This raises concerns about the development of antibiotic resistance.[9] The development of resistance in the pathogenic flora of fish causes further problems for the fish processor, whereas the consumer and health authorities are more alarmed by the possible development of resistance in human pathogens transferred from the microflora of the fish or by the general spread of antibiotic resistance in the aquatic microflora. Several studies show that waste waters from hospitals, farms, and communities increase the antibiotic resistance of enteric organisms found in the water,[107,113] and it has been demonstrated that usage of oxolinic acid and oxytetracycline increased the level of antibiotic resistance in the normal fish microflora.[60,248] Thus, 29% and 27% of 202 isolates from fish farms (where antibiotics were used) were resistant to oxytetracycline and oxolinic acid, respectively, whereas similar values for 94 isolates from an unpolluted stream were, respectively, 6% and 16%.[248]

21.3.2 Fish and Shellfish from Tropical Waters

Wild Fish and Shellfish

The bacterial load on fish caught in clean tropical waters does not differ from counts on temperate fish species (10^2–10^6 cfu g^{-1}, Table 21-8, Table 21-9), however, very high numbers of Enterobacteriaceae (up to 10^7 cfu g^{-1}) can be found on fish caught in polluted warm waters.[97] Despite the warm water, a large proportion (20% to 50%) of the microflora on tropical fish is capable of growth at chill temperatures.[102,197,254]

Shewan[241] concluded that *Bacillus* and *Micrococcus* spp. dominate on fish from tropical waters. Some studies do report high incidences (30% to 50%) of these species[18,253] and tropical waters can contain quite a high proportion (10% to 30%) of Gram-positive organisms.[138] Often, however, the microflora on tropical fish species is very similar to the flora on temperate species (Table 21-6),[6,103,159,172,254] and several authors conclude, as Liston,[173] that the microflora on tropical fish often includes a slightly higher load of Gram-positives and enteric bacteria but otherwise is similar to the flora on temperate water fish.

Several microorganisms pathogenic to humans occur naturally in warm waters; thus most mesophilic vibrios, *Vibrio parahaemolyticus, V. vulnificus*, and *V. cholerae* are found in tropical marine and estuarine waters.[174,223,224] *V. parahaemolyticus* could be isolated year round in tropical Indonesian waters with the lowest incidence (33% positive samples) in December and February and the highest (all samples positive) in July, and the organism was also often isolated from seafood (44% to 100% positive) from the Jakarta area.[191] *V. parahaemolyticus* occurs frequently in the warmer waters around the US with a mean density of 10–20 per 100 ml water.[61] Interestingly, the organism is concentrated in filter-feeding animals, and numbers in oysters are typically 100-

fold higher (up to 5 $\times 10^4$ 100 g^{-1}) than in the surrounding waters.[61] Despite its primary association with the marine environment, *V. parahaemolyticus* can also be isolated from freshwater fish from warm waters. Sakar et al.[234] found an MPN of up to 0.9 g^{-1} in fish from Indian fresh waters and up 90 g^{-1} on plankton and 25% to 30% of newly caught fish were positive for *V. parahaemolyticus*.

Vibrio vulnificus is a mesophilic organism occurring in estuaries in the warm summer months with water temperatures of ≥15°C[205] in which reported disease outbreaks peak.[223] Numbers in the Gulf of Maine ranged from 10^3 100 g^{-1} oyster or ml^{-1} water in July and August (water temperature ~20°C) to below the detection limit in January and February (water temperature <10°C).[205] DePaola et al.,[59] however, found 10^3–10^9 100 g^{-1} or ml^{-1} in fish intestines, oysters, and water from Mobile Bay and Mississippi Sound with the highest numbers found in intestines of bottom fish that fed on molluscs. Such fish and molluscs are obviously a great risk unless cooked or prepared in other ways that eliminate the organisms.

Farmed Fish and Shellfish

As in temperate waters, the microflora on fish and shellfish reared in tropical waters does not differ from the microflora on wild fish.[31,277] However, in some countries in Southeast Asia the fish ponds are enriched with human or animal excreta[273]; for example, chicken manure is used as an organic fertilizer in shrimp farming in Thailand.[57] This obviously raises concern about the level of fecal contamination and several studies have been conducted assessing the prevalence of *Salmonella* and other enteric bacteria in tropical shrimp farming. These organisms do not survive in cold, nutrient-deprived waters, but *Salmonella* and *Esherichia coli* are able to persist and even grow in warm waters.[86,141] Both brackish-water ponds and shrimp from a major producer in Southeast Asia were inherently contaminated with *Salmonella* spp., which were present in 16% of prawns and 22% of mud/water samples from ponds.[217] In contrast *Salmonella* was not isolated from any of 158 samples of water, sediment, shrimp, feed, or chicken manure taken from a Thai shrimp producer[57] and *Salmonella* or fecal coliforms were not detected in Indian cultured prawns.[254]

Several countries require that *Salmonella* are absent from raw, frozen prawns, based on the fact that it is not of marine origin and therefore not a natural part of the microflora. Findings like those cited above show that occurrence of *Salmonella* or *E. coli* cannot be attributed exclusively to poor sanitation or standards of hygiene during processing but are due to conditions during production.[217] Standards, precautions, and preventive measures therefore have to be evaluated according to the production area.

Marine vibrios are found on shellfish and fish reared in tropical waters. Thus *V. cholerae*, all of which were the non-O1 serogroup, were present in 1.5% of prawns and 3.1% of mud/water samples from major aquaculture areas in Southeast Asia.[217] *V. cholerae* non-O1 was isolated from 33% of water, sediment, and shrimp samples from Thai shrimp farms, whereas 2 out of 107 samples were positive for *V. cholerae* O1[57] and *V. cholerae* was detected in Indian cultured shrimp.[196] As in wild fish the incidence of both *V. parahaemolyticus* and *V. vulnificus* was high; 100% and 67% positive samples, respectively.[196]

The problems relating to development and spread of antibiotic resistance are also a concern in tropical aqua- and mariculture; 3 out of 5 *Plesiomonas shigelloides* strains, 4 out of 12 *Salmonella* strains, and 10 out of 13 *Aeromonas hydrophila* isolated from tropical fish farms were resistant to tetracyclines and of a total of 118 isolates, 6% to 75% were resistant to a range of eight different antibiotics.[266]

21.4 MICROBOLOGICAL HAZARDS IN FRESH FISH AND SHELLFISH

The flesh of healthy live fish is generally thought to be sterile as the immune system of the fish prevents the bacteria from growing in the flesh. A notable exception is the muscle of live oysters in which high numbers of *V. vulnificus* are found.[257] When the fish dies, the immune system no longer functions and any bacteria present are able to proliferate freely. The microflora on a fish product will be a function of the natural, indigenous flora (see above) and the microflora typical of the processing environment. The preservation conditions of a given product will typically allow for growth of only a fraction of the microflora and the sections below describe how microorganisms survive, grow, and interact in different fish and shellfish products.

The ability of pathogenic and spoilage microorganisms to grow under different combinations of preservative factors has been studied for many years and during the last decade mathematical quantification of bacterial growth has been used in numerous predictive models.[185] Predictive models, or rather the understanding of microbial ecology on which they are based, is an essential tool in any quality assurance scheme.[187] To date, models evaluate the effect of chemical and physical factors on growth of bacteria and do not take into account interactions between microorganisms in food products.[100]

Spoilage is defined in this context as sensory changes that render the product unacceptable for human consumption. While this occurs for some products as a result of chemical changes, eg, lipid oxidation or bleaching of pigment, the focus of this chapter will be on rejection due to off-odors and off-flavors that are caused by bacterial metabolism. Spoilage of fish cannot be correlated with the total number of mesophilic or psychrotrophic bacteria that develop[38,131] but is often caused by the activities of a minor part of the microflora. An understanding of the specific organisms that are important for spoilage will allow development of better preservation methods and the use of predictive models for determination of shelf life.[51,96,146]

A clear distinction should be made between the terms *spoilage flora* and *spoilage organisms (bacteria)* because the first describes merely the bacteria present on the fish when it spoils, whereas the latter is the specific group that produces the off-odors and off-flavors associated with spoilage. In order to determine which bacteria are the cause of spoilage of fish, the sensory, microbiological, and chemical changes during storage must be measured and the concentration of chemical compound(s) that correlates with spoilage (the chemical spoilage indicator) should be determined. Pure and mixed cultures of bacteria isolated at the time of spoilage are screened in sterile fish substrates for their *spoilage potential,* ie, their ability to produce sensory (off-odors) and chemical changes typical of the spoiled fish.[36,101,111] Selected strains are tested to evaluate their *spoilage activity,* ie, the relation between their growth rate and their *qualitative* and *quantitative* production of off-odors.[52,95] Spoilage potential and activity can be assessed in substrates such as sterile, raw fish juice,[167] heat-sterilized fish juice,[37,52,101] or on sterile muscle blocks.[111] The latter is the most complicated but is most comparable with whole fish.

21.4.1 Raw Bivalve Molluscs

Molluscs are filter-feeding organisms, and many agents in the surrounding waters are concentrated in the animal. They are grown and harvested in shallow, near-shore estuarine waters and are therefore very likely to harbor high concentrations of pathogenic organisms. As they often are eaten raw (with the intestinal tract) or after a very mild heat treatment they constitute a serious health risk.

Several regulations contain bacteriological specifications for quality of waters used for culturing of molluscs. In the US specifications include a limiting MPN count of up to 14 fecal coliform bacteria 100 ml^{-1} water, with no more than 10% of the samples with an MPN count up to 43 fecal coliform bacteria 100 ml^{-1}.[81] However, the value of these indicators is limited for several reasons. First, there is no direct relationship between the microbial content of the filter-feeding bivalve and that of the aquatic environment. Second, the level of indicators does not correlate with the occurrence of human pathogens, eg, *Salmonella,* in the animal. For instance, Martinez-Manzanares et al.[183] found *Salmonella* in 41% of 29 shellfish samples from water with $<10^2$ fecal coliforms 100 ml^{-1} whereas only 29% of 22 shellfish samples from waters with $>10^2$ fecal coliforms 100 ml^{-1} contained *Salmonella.* Such discrepancies may be explained by the investigations showing that *Salmonella* can persist longer in estuarine waters than *E. coli,*[218] obviously raising the question of the suitability of fecal coliforms, for example, as indicator organisms in this environment.[148]

The European Union (EU) has no microbiological standards for waters in which bivalve molluscs are grown but has placed standards on shellfish for direct consumption (see below). The regulation also contains maximum limits for various marine toxins (see Chapter 54) and requires that the growing area is surveyed for toxic phytoplankton.[68]

Molluscs may, at least partly, be cleansed of microbiological contaminants by placing them in clean water in which they actively feed. The relaying procedure involves moving the molluscs to approved shellfish growing waters, whereas depuration is the procedure in which the animals are brought into tanks with clean water of appropriate salinity and temperature. Several countries including Australia, New Zealand, the United Kingdom (UK), Spain, Italy, France, Denmark, Canada, and Turkey require that bivalve molluscs be depurated.[225] While depuration may remove enteric pathogens, neither biotoxins nor chemicals are removed by these procedures and some viruses (eg, hepatitis A) and vibrios persist despite depuration.[135,209,221] Up to 18% to 40% of hepatitis A virus has been found after 5 days of depuration.[135,221] While 10^2–10^3 *E. coli* g^{-1} clam were reduced more than 100-fold by depuration for 24 hours, a similar level of *Vibrio parahaemolyticus* remained constant for 24 hours and decreased to approximately 10% in 3 days.[105] Similar levels of *V. parahaemolyticus* were found in unpurified and depurated (24 hours) Sydney Rock oysters.[74]

Health Hazards

Raw bivalve molluscs (oysters, clams) are very frequently implicated in foodborne disease,[223] thus the majority of all seafood-related diseases in the US are related to consumption of bivalve molluscs.[44] Despite strict regulations for the water quality in the areas in which molluscs are harvested (see above) and various depuration regimes, viral diseases (hepatitis) and gastroenteritis (*V. parahaemolyticus* gastroenteritis), cholera, and recently cases of *V. vulnificus* infection are caused by molluscs.[44] As mentioned in Section 21.1, the largest reported outbreak of foodborne disease was hepatitis A infection involving 290,000 people in China who had consumed undercooked mussels.[258] Until the 1950s, typhoid was frequently transmitted by molluscan shellfish[220,223] but has largely disappeared in the US probably because of fewer cases of waste discharge and improved sewage treatment.[29] A large number (70%) of the outbreaks of disease caused by molluscs in the US (1970–1984) were of unknown etiology.[121]

Hepatitis A and Norwalk viruses are major causes of disease associated with molluscan shellfish. Viruses are able to persist in bivalve molluscs for a longer time than fecal coliform bacteria and these bacteria are therefore not adequate indicators of virus contamination of bivalve molluscs.[220,261] Viruses are heat sensitive and some countries, eg, the UK, require a minimum heat treatment of 90°C for 1 minute for the treatment of cockles from some sources. However, most shellfish are eaten raw or after only a light heat treatment (steaming), which will not affect the viruses.

Several of the health hazards associated with bivalve molluscs are caused by vibrios that are indigenous to the marine environment and their occurrence obviously does not correlate with any of the commonly used indicator organisms.[175] Much focus has recently been on *V. vulnificus*, which causes wound infections or gastroenteritis. In people with liver disease, the mortality rate exceeds 50%. Several attempts have been made, particularly in the US, to devise procedures that would control this organism without altering the sensory charateristics of the product. As *V. vulnificus* is sensitive to various preservative factors, combinations of chilling, lowering pH, mild heating, or irradiation have been suggested.[8,45] However, as the infectious dose is low, this is unlikely to guarantee safety.

Although cholera is mainly a disease associated with contaminated water supplies, *V. cholerae* may also be transmitted by seafood.[29,223] The organism can persist in molluscs and is not spontaneously cleared during depuration.[209] *V. cholerae* is able to survive for long periods in both chilled and frozen products (see Chapter 48). However, because it is heat sensitive, it is mainly a problem of raw foods, particularly bivalves, or if poor hygiene allows for cross-contamination.

Some cases of gastroenteritis caused by cooked oysters have been linked to *Plesiomonas shigelloides*.[230] Although the organism has been isolated from patients with diarrhea, it is not a proven cause of gastroenteritis.[224]

Because the bacteriological quality of bivalve molluscs cannot be predicted from the water quality, the legislative authorities in some countries use end-product testing. The US specifies a limit of <230 MPN fecal coliform per 100 g meat and an aerobic plate count not exceeding 5×10^5 cfu g^{-1}.[81] Similar limits for fecal coliforms are set by the EU that also requires the absence of *Salmonella* in 25 g and set limits for biotoxins.[68] However, unless the bivalves are tested for all potential pathogens, there is no way of guaranteeing their safety and "if people would stop eating uncooked molluscan shellfish and certain types of tropical reef fish, more than 60% of all presently reported seafood-originated illness would not occur."[174]

Spoilage

As most bivalve molluscs are eaten raw, the animal is often alive until just before consumption and the immune system of the organism protects it against spoilage. Chilled molluscs meat removed from the shell ("shucked") has a short shelf life of 5 to 10 days at 1° to 3°C. Little is known about the microbiology during storage, and some authors have suggested that a spoilage dominated by fermentative organisms (lactobacilli, streptococci) takes place.[44] Because of the glycogen content, the pH of the molluscs drops more than that of crustaceans and fish, and the growth of fermentative organisms is therefore likely. However, Kator and Fisher[149] found that although the internal pH of sea scallop dropped to 5.5, the pH on the outer surface remained above 6.0 and increased as storage progressed. The aerobic psychrotrophic count increased from an initial 10^4 cm^{-2} to 10^9 cm^{-2} after 15 days at 2°C. Spoilage was caused by "yellowing" attributed to growth of *Pseudomonas* spp., indicating that the spoilage of chilled molluscs is caused by the same organisms that spoil chilled fish and shellfish (see below).

21.4.2 Fresh and Packed Fish and Shellfish

Fish and shellfish that are distributed fresh receive only very limited processing. The fish or shellfish are caught and in many areas chilled. In some areas of the world, some fish species are eviscerated on board the boats, and in North Europe gutting of white, lean species is compulsory. For instance, cod will deteriorate faster if left ungutted as discoloration and off-odors appear at the belly area after a few days of iced storage.[122] In other fish, such as South American hake, gutting has no influence on quality.[122] Chilling of fish and shellfish is usually done by storage in crushed ice or by covering in chilled seawater (a mixture of ice and seawater). Both procedures will, if done correctly, chill the fish to –1.5° to 1°C. Further processing often involves filleting and skinning before packing and distribution. Fillets (or whole fish) are distributed aerobically or packed under vacuum or in modified atmosphere. The effects of different packing methods on health hazards and spoilage are discussed below.

Health Hazards

Fresh fish of good quality is very unlikely to be the cause of disease if it is not temperature abused and if heated before consumption. The majority of public health hazards related to fresh and packed fish are caused by scombroid toxin and ciguatera toxin (see Chapter 54). Thus, 31% of diseases transmitted by fish in the US from 1970 to 1984 were caused by ciguatera toxin, and 33% were caused by scombroid toxin.[121] Most bacterial disease-causing agents will grow poorly in competition with the natural Gram-negative microflora and, in general, the products will spoil before hazardous levels of organisms or toxin(s) are reached.

Clostridium botulinum. Many studies have investigated the ability of psychrotrophic *C. botulinum*, particularly type E, to grow and produce toxin in fresh fish. Oxygen is inhibitory to *C. botulinum*[71,129] and a review of published experiments showed that fish stored aerobically at chill temperatures (0° to 12°C) spoil before becoming toxic.[214] At higher temperatures (>20°C) spoilage and toxin production in aerobically stored fish sometimes coincided.[214] Concerns have been raised about growth of psychrotrophic nonproteolytic *C. botulinum* in fish packed under vacuum or in modified atmosphere.[80,88] Although the elimination of oxygen during vacuum packing may enhance growth and toxin production by *C. botulinum*,[129] several studies have concluded that only at abuse temperatures will vacuum packed fresh fish become toxic at or before spoilage.[71,215]

Vacuum packed fresh blue crab, tilapia, rainbow trout, flounder, cod, and whiting inoculated with spores of *C. botulinum* type E all spoiled at 4° to 10°C before toxin could be detected (Table 21-7).[88,106,210,215] Lilly and Kautter[171] tested 1,074 portions of commercially packed fresh fish and none became toxic before spoilage. Variable data have been obtained in experiments evaluating whether fish packed under CO_2 become toxic before spoilage. When stored at high temperatures, fish packed in modified atmospheres may become toxic (if inoculated with *C. botulinum* type E) before or at time of sensory spoilage.[214] Eklund[71] found that fresh salmon inoculated with 100 spores of *C. botulinum* type E 100 g^{-1} and stored under 60% or 90% CO_2 at 10°C became toxic within 10 days, and before spoilage. At lower temperatures (0° to 8°C) most studies have found that fresh fish packed in modified atmosphere spoil before containing botulinum toxin (Table 21-7). Eklund[71] similarly concluded that packed (vacuum or modified atmosphere) fresh fish only presents a potential botulinum problem if temperature abused. In contrast to these conclusions, cod inoculated with 50 spores g^{-1}, packed under 100% CO_2 and stored at 4° or 8°C became toxic before being rejected (Table 21-7).[210] Three of 1,016 noninoculated fish packed in modified atmosphere and stored at 8°C were toxic before spoilage was evident. The shelf lives of cod reported[210] are, however, in our opinion and experience unrealistic and shelf life of MAP cod even at 0° to 2°C is normally below 21 days due to spoilage by CO_2-resistant, TMA-producing bacteria.[54] Challenge trials are usually done with inoculated spores at levels of 30–80 g^{-1},[214] whereas the natural contamination is much lower, typically 1–200 kg^{-1},[64] partly explaining why packed fresh fish has never been implicated in outbreaks of botulism. Four conditions must be fulfilled for foodborne botulism to occur:

1. the food must be contaminated
2. spores must survive processing or be able to recontaminate
3. the food must support growth and toxin production
4. the food must be acceptable for consumption without heating sufficient to destroy the botulinum toxin.[71]

The toxins from psychrotrophic *C. botulinum* are heat sensitive and because fresh packed or unpacked fish is cooked before consumed, this is a further safeguard against botulism. Fish used for raw consumption in Japan is extremely fresh, and therefore this poses no extra risk in terms of botulism.

Other Bacterial Pathogens. Many of the diseases transmitted by consumption of raw bivalve mol-

Table 21-7 Time to Sensory Spoilage and Toxin Production by *Clostridium botulinum* type E in Inoculated Packed Fresh Fish Stored at Chill Temperatures ≤10°C

				Days Until		
Fish	*Atmosphere*	*Temperature °C*	*Inoculum**	*Spoilage*	*Toxicity*	*Reference*
blue crab	vacuum	4	10^3–10^4	9	21	106
tilapia	vacuum	4	10^2	47	>90	215
rainbow trout	vacuum	4	10^3–10^4	12	>21	88
tilapia	vacuum	8	10^2	10	17	215
flounder	vacuum	8	50	7	>21	210
whiting	vacuum	8	50	10	17	210
cod	vacuum	8	50	16	20	210
blue crab	vacuum	10	10^3–10^4	6	15	106
rainbow trout	vacuum	10	10^3–10^4	3	10	88
tilapia	CO_2	4	10^2	80	>90	215
whiting	CO_2	4	50	15	27	210
cod	CO_2	4	50	40–53	18–21	210
tilapia	CO_2	8	10^2	17	40	215
flounder	CO_2	8	50	10	23	210
whiting	CO_2	8	50	7–15	5–20	210
cod	CO_2	8	50	16–23	8–19	210
salmon	CO_2	10	10^2	10	10	71

*Spores of *C. botulinum* type E g^{-1}.

luscs can also be transmitted by raw fish. However, it should be emphasized that, as opposed to the bivalve molluscs, there is no accumulation of the pathogenic agent(s) in the fish. An outbreak of cholera in Peru was caused by consumption of ceviche (a lemon-marinated raw fish).[209] As a result, a number of Western countries prohibited the importation of fish products from South America. This may have been a dramatic overreaction, because *V. cholerae* occurs widely in the marine environment and because the excellent sanitary infrastructure in Europe will not allow an isolated incident to spread.[121,193] In general, vibrios are common causes of foodborne disease in countries such as Korea and Japan where large amounts of raw fish are consumed,[163] and in particular *V. parahaemolyticus* gastroenteritis has been linked to consumption of raw fish in Japan.[224]

Pathogenic bacteria able to grow at low temperatures, eg, *A. hydrophila*, *Y. enterocolitica*, and *L. monocytogenes* will all be outgrown by the Gram-negative spoilage microflora during chilled, aerobic storage of fish. Concerns have been raised about the risk of growth of these organisms in fish packed in modified atmosphere; however, several studies have shown that CO_2 at low temperature efficiently inhibit growth on fish of all three organisms.[58,211]

Biogenic Amines (Histamine, etc). Biogenic amines have specifically been associated with fish from the scombroid family (tuna, mackerel) but can occur also in other fish species like mahi-mahi or sardines. The amines are formed by bacterial decarboxylation of various amino acids and many bacteria are capable of producing biogenic amines.[251] Members of the Enterobacteriaceae, particularly *Morganella morganii*, are by far the most potent producers and the decarboxylation normally takes place only at high temperature. Klausen and Huss[150] showed that histamine was not formed in mackerel stored at 0°C but more than 500 ppm could be detected after 3 days at 10°C. However, histamine was also formed in fish stored at 0°C after temperature abuse (48 hours at 10°C) although numbers of histamine-forming bacteria did not increase further. This indicates that the preformed enzyme may be active at low temperatures. Other studies have shown histamine formation at low temperatures[3] and this may be attributed to activity of psychrophilic photobacteria that have been shown to decarboxylate histidine.[192,271]

Parasites. Some of the parasites present in the aquatic environment are pathogenic to man and their presence in fish is both a safety hazard and an aesthetic problem. Most of the parasites have complex life cycles passing through several intermediate hosts[27] including gastropods or crustaceans, and infect humans ingesting fish feeding on these infected organisms. Studies of parasitic infections related to seafood have primarily focused on nematodes in the *Anisakidae* family, which covers species such as *Anisakis simplex* (herring worm), and *Pseudoterranova decipiens* (cod worm). However, cestodes (tapeworms) and trematodes (flukes) have also been involved in human infection.[121,204] It is estimated that 40 million people throughout the world, particularly in Southeast Asia, are affected by trematode infections primarily caused by consumption of raw plants or raw/undercooked freshwater fish.[4] In general, parasitic infections are caused by consumption of raw (unheated) and undercooked fish products. Pond hygiene (eg, removal of intermediate hosts like snails) is important in prevention of parasitic infections from aquaculture fish. Most of the parasitic population is found in the visceral organs, which normally are removed before the fish is consumed. Roepstorff et al.[227] found that 95% to 98% of herring viscera were infected with *Anisakis simplex*, whereas only 2% to 6% and 0% to 1% of belly flaps and fillets were infected, respectively. Some studies show that the nematodes migrate from the intestines into the flesh during storage and gutting is therefore important in prevention of disease. However, no migration was detected in heavily infected herring stored in ice, in chilled seawater, or at 10°C for up to 5½ days.[227] EU[69] requires that fish are visually inspected for parasites and that fish used for particular products are frozen for 24 hours at –20°C (see below). Cooking will eliminate the risk, as the larvae are killed by exposure to 60°C for 1 minute[204] or even by 55°C for 1 minute (Huss unpublished data).

Spoilage

Microbiology of Fish and Shellfish Stored in Ice. On the surface of fish, bacteria colonize the scale pockets and during storage they invade the flesh by moving between the muscle fibers. Murray and Shewan[195] found that a very limited number of bacteria invaded the flesh during iced storage, and bacteria have been detected by microscopy in the flesh when the number of organisms on the skin surface increased above 10^6 cfu cm^{-2}.[228] Because only a limited number of organisms actually invade the flesh and microbial growth mainly takes place at the surface, spoilage is probably to a large extent a consequence of bacterial enzymes diffusing into the flesh and nutrients diffusing to the outside.

Bacteria on fish caught in temperate waters enter the exponential growth phase almost immediately after the fish have died.[111,146] This is probably because the microflora is already adapted to the chill temperatures. During ice storage, the aerobic count increases with a doubling time of approximately 24 hours and will, after 2 to 3 weeks, reach numbers of 10^8–10^9 cfu g^{-1} flesh or cm^{-2} skin. The bacteria on fish caught in tropical waters often pass through a lag phase of 1 to 2 weeks if the fish are stored in ice, thereafter exponential growth begins and the numbers reach a level similar to that on temperate water fish when spoiled.[97,102,182,255] The delay before growth of psychrotrophs on iced, stored tropical fish is one of the factors explaining the long shelf life of these fish stored in ice.[95]

The composition of the microflora changes dramatically during storage. Thus, during aerobic iced storage, the microflora is composed almost exclusively of *Pseudomonas* spp. and *S. putrefaciens*. This is true for all fish and shellfish whether caught or harvested in temperate[101,169] or subtropical and tropical waters (Table 21-8).[40,103,159,172,238]

S. putrefaciens is very important as a spoilage bacterium of iced fish (see below). The bacterium was originally associated with the *Achromobacter* group but was later placed in the *Pseudomonas* group IV.[243] Based on percentage of guanine + cytosine (GC%) it was transferred to the genus *Alteromonas*,[162] but on the basis of 5S RNA homology it was reclassified to a new genus, *Shewanella*.[180] *S. putrefaciens* has long been recognized as a very heterogenous species in general comprising the following two large groups: (1) psychrotrophic organisms, unable to grow in 6% NaCl found on fish and in cold environments, and (2) mesophilic, halotolerant organisms growing in 6% to 8% NaCl. The latter group can occur as opportunistic pathogens causing wound infection and bacteremia.[28,65,170,219] Recently, it was suggested that the mesophilic group be reclassified in its own species, *Shewanella alga*.[84,201] *S. alga*, apart from being an infectious organism, is also of interest in fishery science due to its possible ability to produce the pufferfish toxin, tetrodotoxin.[245]

S. putrefaciens is the specific spoilage bacterium of marine temperate water fish stored aerobically in ice and the number of *S. putrefaciens* is inversely linearly related to remaining shelf life of iced cod[146] (Figure 21-2). *S. putrefaciens* usually constitutes 1% or less of the microflora of fresh fish[39] but increases relative to the aerobic count and constitutes 30% to 90% of the microflora at point of spoilage.[39,146]

Table 21-8 Changes in Composition of Surface Microflora during Iced Storage of Fish and Shellfish from Tropical Waters[6,19,31]

	Percentage Distribution of Microflora											
Species	*Mackerel* (Rastrelliger faughni *Matsui)*					*Tilapia* (Tilapia aurea*)*					*Shrimp* (Penaeus*)*	
Days of Storage	0	1	5	10	15	0	3	9	15	19	2	16
Total no. per gram	10^4	10^5	5×10^5	5×10^7	10^9	2×10^4	2×10^2	10^3	5×10^6	6×10^7	8×10^4	2×10^8
Pseudomonas	30	25	38	58	53	16	27	62	82	71	—	87
Shewanella putrefaciens	0	0	38	14	42						—	4
Acinetobacter/ Moraxella/ Alcaligenes	10	8	—	—	—	19	11	6	16	15	15	4
Flavobacterium						13	1	7	—	—	20	—
Vibrionaceae						16	1	6	—	—	—	1
Enterobacteriaceae	10	—	6	—	—	—	4	—	—	—	—	—
Bacillus	40	42	18	14	—	1	13	—	1	6	8	—
Micrococcus a.o.						26	22	3	—	2	38	—
Corynebacterium	10	17	—	—	—	4	22	16	1	6	13	—
Unknown/other	—	8	—	14	5	5					6	4

Pseudomonas spp. are the specific spoilers of iced stored tropical freshwater fish[103,172] and are also, together with *S. putrefaciens*, spoilers of marine tropical fish stored in ice[40,91,97] (Tables 21-8 and 21-9). *S. putrefaciens* has been isolated from tropical fresh waters, but does not appear to be important in the spoilage of iced freshwater fish from tropical waters.[103,172] This may be due to occurrence of very low numbers and the inability of the organism to compete with high numbers of antagonistic pseudomonads.[99,100]

Vacuum packing and subsequent iced storage of fish from temperate marine waters reduces the number of *Pseudomonas* spp. but *S. putrefaciens*, which is capable of anaerobic respiration using TMAO as electron acceptor, grows to levels of 10^5–10^8 cfu g^{-1}.[55,146] Numbers below 10^8 cfu g^{-1} are unlikely to be important in spoilage and consequently other organisms must be involved. Jørgensen et al.[146] observed that vacuum packed cod contained high numbers of some very large, almost yeast-like cells and suggested that these were involved in the spoilage. These cells are *P. phosphoreum*,[55] a marine vibrio that has been overlooked because it is killed by exposure to temperatures of 45°C used in pour plating.[269] It is easily isolated from intestines of various fish.[52,269] The organism has a very high spoilage activity, producing between 10- and 100-fold more TMA per cell than *S. putrefaciens*[52] but does not cause such foul off-odors as *S. putrefaciens*,[270] probably because it does not produce volatile sulfides.[51,55,270] The spoilage of vacuum packed fish from temperate marine waters is caused by these two bacteria and differences in initial numbers of *S. putrefaciens* and *P. phosphoreum* probably decide which of the two becomes most important. While abundant data exist on marine fish, little is known about the spoilage bacteriology of vacuum packed freshwater fish. It is unlikely that *P. phosphoreum* plays a major role in the spoilage of freshwater fish as it is sodium requiring. Hussain et al.[132] found while the initial flora (10^4 cfu g^{-1}) on trout was dominated by Gram-negative organisms (*Pseudomonas, Moraxella, Flavobacterium*), 4 weeks of iced storage of vacuum-packed trout selected for Gram-positive bacteria (*Micrococcus, Bacillus, Lactobacillus*), which reached 10^8 cfu g^{-1}. However, pour plating was used in this work, eliminating any very heat-sensitive organisms.

CO_2 packing of marine fish from temperate waters inhibits growth of organisms like *Pseudomonas* and *S. putrefaciens* and their numbers rarely exceed 10^5–10^6 cfu g^{-1}. Similar changes are seen for meat products[50] and result in dramatic extensions of shelf life. However, compared with meat no or only limited extension of shelf life is obtained by packing fish in a CO_2 atmosphere.[33,34,55,80] The development of TMA in CO_2 packed cod is, as shown in Figure 21-3A, enhanced compared with that in aerobic storage.[33,55] The presence and subsequent growth of CO_2-resis-

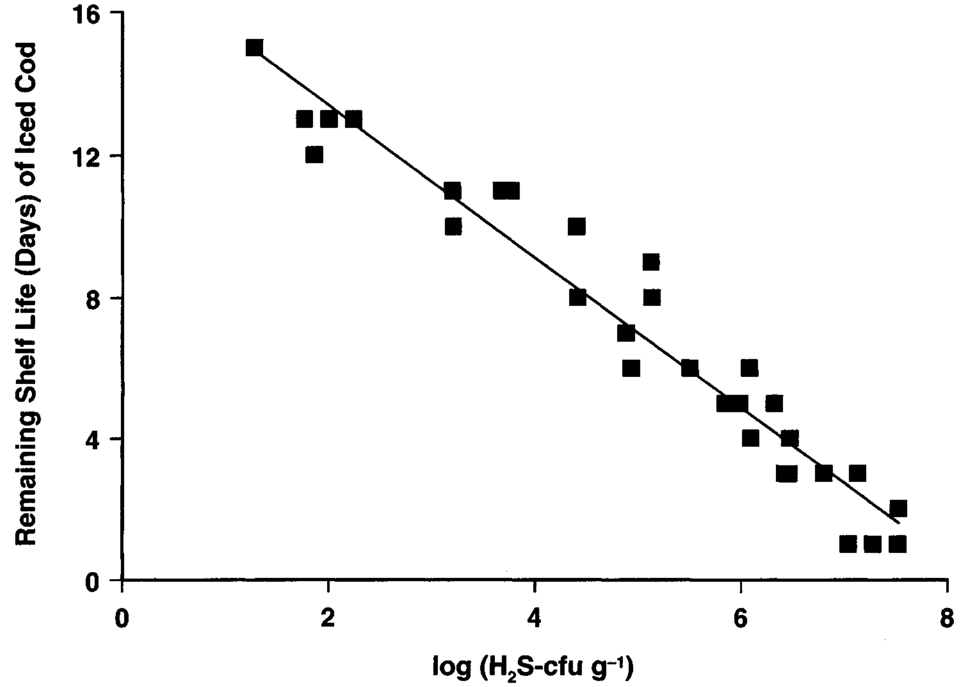

Figure 21-2 Correlation between Remaining Shelf Life of Iced Cod and Numbers of H_2S Producing Bacteria Determined on Iron Agar. *Source:* Adapted with permission from B.R. Jorgensen, D.M. Gibson, and H.H. Huss, Microbiological Quality and Shelf Life Prediction of Chilled Fish, *International Journal of Food Microbiology,* Vol. 6, pp. 295–307, © 1988, Elsevier Applied Science.

tant TMA-producing *P. phosphoreum* to levels of 10^7–10^8 cfu g^{-1} explains these observations.

In iced fish stored aerobically, spoilage is associated with the development of 10^8–10^9 cfu g^{-1} of specific spoilage bacteria. In chilled fish packed under CO_2, spoilage occurs with 10^7 *P. phosphoreum* g^{-1}.[55] This is because the very large size (5 μm) of the bacterium results in a much higher yield of TMA per cell than for *S. putrefaciens*[52] and thus a higher spoilage activity.

Carbon dioxide and vacuum packing of fish caught in freshwater or warmer waters where these particular heat-sensitive, sodium-requiring *P. phosphoreum* are probably not as common as in temperate marine waters result in decrease in TMA production (Figure

Table 21-9 Distribution of Gram-Positive and Gram-Negative Bacteria on Swordfish from Gulf of Mexico Stored at 3.5°C with and without CO_2

	Percentage Distribution of Microflora									
Bacterial Group	*Fresh*	*Aerobic*				*40% CO_2/60% N_2*				
Days of Storage	0	2	5	8	11	2	5	8	14	18
Total Count	4×10^4	7×10^4	5×10^5	10^7	9×10^7	5×10^3	10^4	4×10^4	2×10^6	4×10^6
Gram-negative	95	92	100	100	93	96	97	35	4	0
Pseudomonas spp.	26	75	100	100	92	55	94	17	4	0
Gram-positive	5	8	0	0	7	4	3	65	96	100
Lactobacillus spp.	0	0	0	0	0	0	0	39	55	100

Source: Adapted with permission from M. Lannelongue et al., Microbiological and Chemical Changes During Storage of Swordfish (*Xiphias Gladius*) Steaks in Retail Packages Containing CO_2-enriched Atmospheres, *Journal of Food Protection,* Vol. 45, pp. 1197–1203, © 1982, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

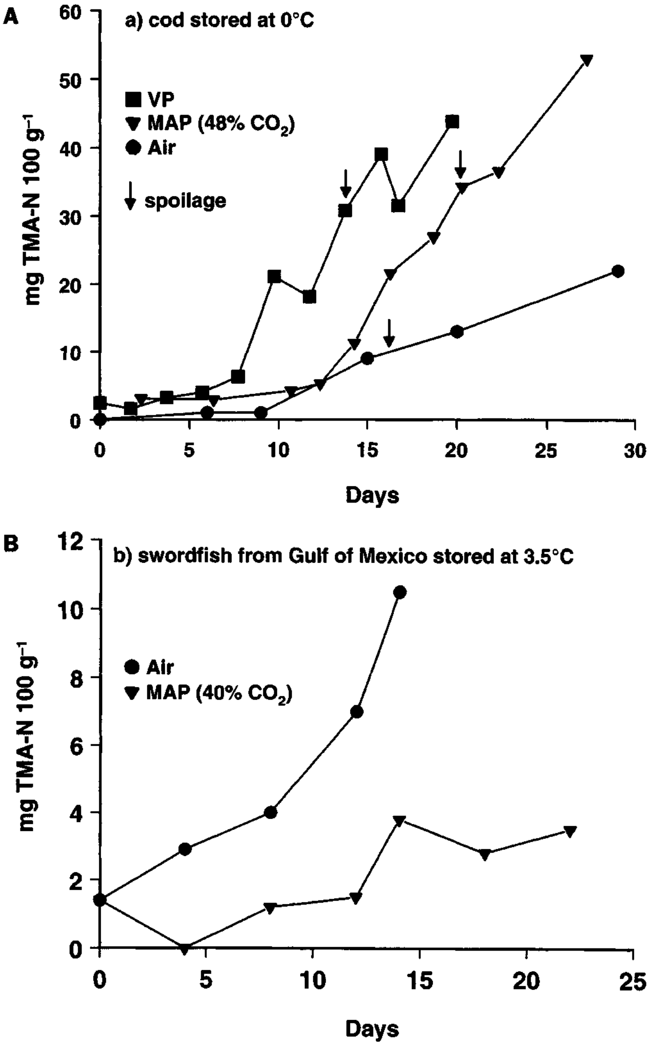

Figure 21-3 TMA Production during Chill Storage of Marine Fish from (A) Temperate and (B) Tropical Waters. **A**, Cod Stored under Air (●), Vacuum-Packed (■), or in 48% CO_2/52% N_2 (▼) at 0°C; ↓ Indicates Spoilage.[55] **B**, Swordfish from the Gulf of Mexico Stored in Air (●) and in 40% CO_2/60% N_2 (▼) at 3.5°C. *Source:* Adapted with permission from M. Lannelongue et al., Microbiological and Chemical Changes During Storage of Swordfish (*Xiphias Gladius*) Steaks in Retail Packages Containing CO_2-enriched Atmospheres, *Journal of Food Protection,* Vol. 45, pp. 1197–1203, © 1982, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

21-3B) and the microflora becomes dominated by various Gram-positive organisms, mainly lactic acid bacteria.[18,159,160,202,207]

The changes in distribution between Gram-negative and Gram-positive microorganisms in CO_2 packed fish from warm waters are shown in Table 21-9. In some trials, TMA can be detected later in the storage,[202,213] and therefore some TMAO-reducing organisms must be present. Table 21-10 gives a summary of the specific spoilage bacteria of chilled stored fish from both temperate and tropical waters.

Microbiology of Fish and Shellfish Stored at Ambient Temperatures. During ambient storage of tropical fish and shellfish, a level of 10^7–10^9 mesophilic cfu g^{-1} is reached in 12–24 hours.[92,101,102] The microflora is dominated by mesophilic *Vibrio* or *Aeromonas* spp.[92,103,238] and, particularly if the fish are caught in polluted waters, mesophilic Enterobacteriaceae.[97] At ambient temperature, motile aeromonads are the specific spoilers of aerobically stored freshwater fish.[16,92,103] A large proportion of the flora on ambient-stored mackerel consisted of *S. putrefaciens,* indicating that this bacterium may also take part in the spoilage.[19]

Biochemical Changes Caused by Bacterial Action. Comparison of the chemical compounds that develop in naturally spoiling fish and sterile fish has shown that most of the volatile compounds are produced by bacteria.[239] These include TMA, volatile sulfur compounds, aldehydes, ketones, esters, hypoxanthine as well as other low molecular weight compounds. The substrates for the production of volatiles are TMAO, sulfur-containing amino acids, carbohydrates (eg, lactate and ribose), nucleotides (eg, inosine monophosphate and inosine), and other NPN molecules. The amino acids are particularly important substrates for formation of sulfides and ammonia (Table 21-11).[109,110,222] Lerke et al.[168] separated fish juice into a protein and a nonprotein fraction and found that the nonprotein fraction of a fish juice spoiled in the same way as the whole juice, whereas only faint off-odors were detected in the protein fraction of the juice. Although some authors have used the number of proteolytic bacteria as indicators of spoilage, the turnover of the protein fraction is not of major importance in spoilage of fresh fish. The initial aerobic growth on fish is dominated by bacteria using carbohydrates as substrate and oxygen as terminal electron acceptor with the concurrent production of CO_2 and H_2O. This results in the formation of anaerobic or microaerophilic niches on the fish. This does not, however, necessarily favor the growth of anaerobic bacteria as some of the bacteria present on fish are able to carry out a respiration using other molecules as electron acceptor. It is typical that many of the specific spoilage bacteria on fish are able to use TMAO as electron acceptor in an anaerobic respiration.

Table 21-10 Specific Spoilage Bacteria of Fresh and Packed Fish Stored Chilled (<4°C) or in Ice

Storage Conditions	*Specific Spoilage Organisms of Fresh, Chilled Fish Depending on Source of Fish*			
	Temperate Waters		*Tropical Waters*	
	Marine	*Fresh*	*Marine*	*Fresh*
Aerobic	*S. putrefaciens*[111,145,169]	*Pseudomonas* spp.*	*S. putrefaciens*[91,97]/ *Pseudomonas* spp.[91,97]	*Pseudomonas* spp.[103,172]
Anaerobic	*S. putrefaciens*[55,146]/ *P. phosphoreum*[55,146]	Gram-positive bacteria[132] Lactic acid bacteria*	Lactic acid bacteria[207]/ others ?	Lactic acid bacteria ?
CO_2 (20% to 70%)	*P. phosphoreum*[52,55]	Lactic acid bacteria*	Lactic acid bacteria[18,159,202]/ TMAO-reducing bacteria	Lactic acid bacteria ?/ TMAO-reducing bacteria[213]

*Assumed to be the most likely spoilage bacteria as typical marine bacteria are not present.

Trimethylamine (TMA). A major aspect of fish spoilage is the reduction of TMAO to trimethylamine (TMA) by microorganisms, resulting in the typical "fishy" odor of spoiled fish. Spoilage of fish by reduction of TMAO is mainly caused by *S. putrefaciens, P. phosphoreum, Vibrio,* and *Aeromonas* spp. and members of the Enterobacteriaceae (Table 21-11).[66,222,233,236,249] The ability of bacteria to reduce TMAO and/or produce H_2S can be tested in specific media and/or with specific methods.[101,162,269]

Sulfides. Many bacteria identified as the cause of spoilage produce one or several volatile sulfides. *S. putrefaciens* and some *Vibrionaceae* produce H_2S from the sulfur-containing amino acid L-cysteine.[101,250] In contrast, neither *Pseudomonas* nor *P. phosphoreum* produces significant amounts of H_2S. Thus, hydrogen sulfide, which is typical of spoiling iced cod stored aerobically, is not detected in spoiling CO_2 packed cod.[55] Some lactobacilli growing in lightly preserved fish products (eg, *Lactobacillus saké*) produce H_2S.[20,263] Methyl mercaptan (CH_3SH) and dimethyl sulfide (($CH_3)_2S$) are both formed from methionine.[109] Taurine, which is also sulfur containing, occurs as free amino acid in very high concentrations in fish muscle and disappears from the fish flesh during storage but this is believed to result from leakage rather than bacterial attack,[242] although both pseudomonads[244] and several members of Enterobacteriaceae may catabolize taurine.[268] Spoilage by *Pseudomonas* spp. results in fruity, rotten, sulfhydryl odors[91,102,103] because of formation of aldehydes, ketones, esters, and sulfides.[67,189,190] The fruity off-odors produced by *Pseudomonas fragi* originate from monoamino-monocarboxylic amino acids.

Hypoxanthine. The development of TMA is in many fish species parallelled by a production of hypoxanthine (Hx). Hx has a slightly bitter flavor, and can be formed by the autolytic decomposition of nucleotides, but can also be formed by bacteria; the rate of bacterial formation is higher than that by autolysis. Both Jørgensen et al.[146] and Dalgaard et al.[55] showed a linear correlation between the contents of TMA and Hx during iced storage of packed cod. Several of the spoilage bacteria including *Pseudomonas* spp.,[256] *S. putrefaciens,*[95,145,270] and *P. phosphoreum*[270] produce Hx from inosine or inosine monophosphate. While Hx does not cause spoilage off-odors, it may be used as a spoilage index in some fish.

Detection of Specific Spoilage Bacteria from Fresh and Packed Fish. Several methods have been developed to enumerate spoilage bacteria from fish and shellfish.[98] The ability of certain spoilage organisms, particularly *S. putrefaciens,* to produce H_2S (Table 21-11) has been used in several media in which H_2S is precipitated with ferrous ions as black FeS.[101,169] When iced fresh fish is investigated, the majority of bacteria forming black colonies on Iron Agar are *S. putrefaciens,*[52,101,145] although *Vibrionaceae* capable of forming H_2S from cysteine can also occur, especially in the warm summer months.[101,103] The ability of *S. putrefaciens* to form a red/brown pigment has been used in the gelatine-iron medium of Long and Hammer[176] and in a modified version[269] also suitable for enumeration of *P. phosphoreum,* which appeared as small frosted glass-like colonies.

Reduction of TMAO is used in the conductometric quantification of spoilage bacteria.[90,94,146] The de-

Table 21-11 Substrate Used and Typical Spoilage Compounds Produced by Bacteria during Storage of Fresh and Packed Fish

	Production (+) of Spoilage Compounds						
Substrate	*TMAO*	*Cysteine*	*Methionine*	*Other Amino Acids*	*IMP, Inosine*	*Carbohydrates, Lactate*	
Compounds	*TMA*	*H_2S*	*CH_3SH, $(CH_3)_2S$*	*Ketones, Esters, Aldehydes, NH_3*	*Hypoxanthine*	*Acids*	*Product Examples*
Spoilage Bacteria							
S. putrefaciens	+	+	+	?	+	+	iced marine fish
Pseudomonas spp.	–	–	+	+	+	?	iced freshwater fish
P. phosphoreum	+	–	–	?	+	?	CO_2 packed fish
Vibrionaceae	+	+	?	?	?	?	ambient stored fresh fish
Enterobacteriaceae	+	(+)	+	+	+	+	lightly preserved fish
Lactic acid bacteria	–	(+)	?	+	?	+	lightly preserved fish
Yeast	–	–	–	+	?	+	sugar-salted fish
Anaerobic rods	–		?	+	?	?	*sous-vide* fish

tection times (DTs) are typically below 24 hours and the DTs in a TMAO-containing medium were inversely proportional to the number of black colonies on Iron Agar.[94,146] A conductometric method based on TMAO reduction for specific detection of *P. phosphoreum* was recently developed; selectivity is obtained by simulating the conditions in packed fish, ie, using CO_2 incubation.[56]

Monoclonal antibodies against *S. putrefaciens* have been developed with the purpose of establishing a rapid detection method.[83] However, the lower detection limit of immuno-based methods is 10^6 cfu g^{-1}. Several detection schemes have recently, with success, used immunomagnetic capture to concentrate bacteria from a sample. We have tried to use *S. putrefaciens* specific antibodies in such a procedure. Unfortunately, the general excellent ability of fish bacteria to adhere to surfaces has made it impossible to prevent nonspecific binding of bacteria to the magnetic bead, and thereby to achieve a *specific* capture of the *S. putrefaciens*. An rRNA probe for specific detection of *S. putrefaciens* has been developed,[62] however, this has not been evaluated using *S. putrefaciens* of fish origin.

21.5 MICROBIOLOGICAL HAZARDS IN FISH AND SHELLFISH PRODUCTS

Storage of fresh fish at various temperatures almost exclusively selects for Gram-negative bacteria (except for packed tropical fish) and the specific spoilage bacteria and their metabolism has been extensively studied. The combination of chilling with the addition of low levels of salt and chemical preservatives (lightly preserved fish products) causes a dramatic shift in the microbiology and both microbiological health hazards as well as microbiological spoilage become much more complex as several groups of organisms may grow and interact. Particularly, the spoilage of this type of product is not well understood. The microbiology of more heavily preserved fish products (eg, heavily salted or heated products) is, compared with fresh fish, better understood, perhaps because only few bacterial species are able to survive and grow.

21.5.1 Lightly Preserved Seafood Products

This group includes fish products preserved by low levels of salt (<6% [w/w] NaCl in the water phase) and, for some products, addition of preservatives (sorbate, benzoate, NO_2^-, or smoke). The pH of the products is high (>5.0), and they are often packaged under vacuum and must be stored and distributed at chill temperatures (≤5°C). This is a group of high-value delicatessen products (cold-smoked, pickled ["gravad"], or marinated fish, brined shellfish) that are typically consumed as ready-to-eat products with no heat treatment.

Cold smoking of fish involves salting (pickling, brining, or more common today, salting by injection), drying for 1 to 6 hours at 20° to 28°C and smoking at a maximum of 30°C for 3 to 6 hours.[130] The products usually have a shelf life of 3 to 6 weeks at 5°C. Gravad fish is a typical Scandinavian product where fish fillets (salmon, halibut, trout) are sprinkled with salt, sugar, and spices and left to ripen for 1 to 2 days at chill temperatures. Often this

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product is made at home without control of salt levels or temperature; particularly homemade gravad fish has been implicated as a vehicle for botulism.[121] Brined products are typically made from cooked crustaceans (shrimps, crabs, lobster meat), which are peeled and put into a brine with salt, carbohydrate, and preservatives such as sorbate and benzoate. Glucose has been used but may result in slime formation due to growth of *Leuconostoc* spp., whereas sugar alcohols give the sweet flavor but cannot be used as substrate in slime formation.

As some psychrotrophic pathogenic bacteria are able to survive and grow in lightly preserved fish products, the fish industry and consumers have recently focused on alternative means of preservation, eg, the use of bioprotective cultures, bacteriocins, enzymes, or cationic peptides. The bacteria or compounds are used to inhibit or delay the growth of pathogens or spoilers *without* affecting the sensory attributes of the product. As described below, particularly the presence and growth of *L. monocytogenes* in lightly preserved fish products is of major concern and use of lactic acid bacteria,[140] lysozyme,[142] protamine,[143] or nisin[200] have all proved successful in preventing or delaying the growth of this organism under conditions mimicking those in lightly preserved fish products or in real products.

Health Hazards

Lightly preserved fish products constitute, next to the raw bivalve molluscs, the most dangerous group of fish products.[121] Almost any of the pathogenic organisms listed in Table 21-2 may be transferred via these types of products. The occurrence of the indigenous organisms obviously cannot be prevented. However, prevention of disease relies on inhibition of growth by keeping levels of the contaminants low, ensuring a proper salt concentration, and distributing at chill temperatures.

Clostridium botulinum. The psychrotrophic nonproteolytic type B, E, and F strains are, under otherwise optimal conditions, able to grow at temperatures above 3.3°C and in low salt concentrations (<5% NaCl).[120] However, in chilled fish products (5°–10°C) less salt is needed to inhibit growth than at higher temperatures (Table 21-12). Also, pH interacts with NaCl (or water activity [a_w]), thus at 20°–30°C, *C. botulinum* Type E germinated and grew at a_w of 0.96 (approx. 5% NaCl) at pH 7.0, whereas the tolerance was only a_w 0.98 (approx. 3% NaCl) at pH 5.5.[17] High inocula (10^7–10^8 spores ml^{-1}) were used in this study. Using a lower inoculum of 10^4–10^5 spores of nonproteolytic *C. botulinum* ml^{-1} meat medium with 2.5% NaCl, it was similarly found that at 5°C growth occurred after 24 to 27 days at pH 6.5 and not until after 31 to 47 days at pH 5.6.[93] Vacuum packing enhances toxin production in smoked fish[129] but, as mentioned, the positive redox potential of fish may be an additional hurdle preventing growth in salted fish.[129] Several incidents of botulism associated with commercially produced smoked vacuum packed fish have been reported, but in all cases the packed fish was temperature abused.[5]

Overall, the combination of low temperature (≤5°C), a minimum of 3% to 3.5% NaCl in the water phase, and—for brined shrimp—the reduction in pH to 5.5 ensures the safety of lightly preserved fish products with respect to botulism. In over 75% of the incidents in which botulism has been caused by fish products, these have not been produced commercially.[186] Where commercial products have been involved, this has been due to post-process contamination of canned

Table 21-12 Toxin Production by *Clostridium botulinum* type E at Chill Temperatures Depending on Salt Concentration

Temperature, °C	*% NaCl (WPS)*	*Inoculum (spores g^{-1})*	*Substrate*	*Days Till Toxicity*	*Reference*
5	2.0	n.i.[a]	soy peptone broth	55	186
	2.5	10^4–10^5	meat medium	24–27	93
	3.0	n.i.	soy peptone broth	60[b]	186
8	2.5	10^4–10^5	meat medium	8–9	93
10	2.0	n.i.	soy peptone broth	25	186
	2.5	not inoculated	hot-smoked fish	>30	32
	2.5	10^2	hot-smoked fish	<30	32
	3.0	10^2	hot-smoked fish	>30	32

[a] n.i. = not indicated; the study was performed with a mixture of nonproteolytic *C. botulinum* strains.

[b] As predicted from a response surface model.

fish, temperature abuse, or to faulty salting and/or fermentation (see Sections 21.5.2 and 21.5.3).

Listeria monocytogenes. Much research has been directed to the study of occurrence and growth of *L. monocytogenes* in lightly preserved fish products. The organism is a frequent contaminant in the raw material;[130] it survives the salting and cold-smoking process (≤30°C), and it is able to grow in the finished product at chill temperature.[23,118,140,231] Recently, six cases of listeriosis in Wärmland in Sweden were linked to the consumption of cold-smoked fish products; several typing methods showed that strains isolated from two of the patients were identical to strains isolated from the cold-smoked and gravad rainbow trout.[73]

L. monocytogenes may be found on up to 75% of cold-smoked salmon and other lightly preserved fish products.[21] Rawles et al.[212] reported that 13 of 126 freshly cooked blue crab samples were positive for *L. monocytogenes* with levels of fewer than 100 g^{-1}, and other studies similarly report low levels.[80,130] Danish investigations have recently found that 24% of freshly produced (and packed) cold-smoked salmon were positive for *L. monocytogenes* with 18% containing less than 10 g^{-1} and 6% containing between 10 and 100 g^{-1}.[123,144] An important prerequisite for control of *L. monocytogenes* in these products is knowledge about the contamination route. A high correlation was found between occurrence of *L. monocytogenes* in the seawater and on the raw material.[130] Low prevalence of *L. monocytogenes* was found in three Swiss fish farms with 9 of 36 water samples positive and only 5 of 45 fish samples positive.[137] None of 27 samples taken immediately after cold smoking was positive for *L. monocytogenes*, and only 1 of 49 samples of finished product was positive. Using multilocus enzyme electrophoresis, Rørvik et al.[232] found that the *L. monocytogenes* strains isolated from cold-smoked salmon were different from the isolates from seawater, indicating that the contaminants could not be linked to the raw material. However, other studies have concluded that the raw material is the most likely contamination source although a clear contamination route has not been found.[72] As the source of contamination is not known and as the preserving conditions do not prevent growth, no critical control point giving full control can be identified for the hazard.[130]

Numerous studies have shown that inoculated *L. monocytogenes* grow well in cold-smoked salmon at chill temperatures (4°–5°C) with 1–2 log increase per week.[231] However, in a review of published data, it was concluded the growth of naturally occurring *L. monocytogenes* in cold-smoked fish is often much slower than predicted by models.[53] At the end of commercial shelf life (storage at 5°C), 31% of samples of cold-smoked salmon were positive for *L. monocytogenes* with 27% having fewer than 10^2 g^{-1}, 2% between 10^2 and 10^3 g^{-1}, and 2% between 10^3 and 10^4 g^{-1}.[123,144] This indicates that the naturally occurring *L. monocytogenes* grew much more slowly than inoculated strains. Jay[134] suggested that the harmless microflora could prevent the proliferation of pathogens, and certainly lightly preserved fish products become dominated by lactic acid bacteria highly antagonistic against *L. monocytogenes.*[139]

Complete elimination of *L. monocytogenes* from this type of product cannot be achieved,[12] but prevention of foodborne disease can be obtained by keeping the number of *L. monocytogenes* very low. This can be done by observing strict Good Manufacturing Procedures.[87,137] A recently proposed microbiological criterion for *L. monocytogenes* in cold-smoked salmon using a three class attribute sampling plan, is $n = 5$, $m = 10$ g^{-1} and $M = 100$ g^{-1} and $c = 1$.[130] This contrasts to the US where a "zero tolerance" (absence in a 50 g sample) is the current limit for ready-to-eat products, although, based on recent assessment of the widespread alimentary exposure to *L. monocytogenes*, it has been suggested that some relaxation of this limit may be possible, but that any change would have to be carefully monitored.[114]

Aeromonas *spp.* Motile *Aeromonas* spp. (*Aeromonas hydrophila*), which occur naturally in the aquatic environment, are able to grow at chill temperatures and can frequently be isolated from fish products.[156] It has been speculated for years that the motile aeromonads may be implicated in foodborne disease,[152,158,188] but convincing epidemiological evidence is lacking. To our knowledge only one report[7] documents that an isolate from a patient with diarrhea was identical, by ribotyping, to strains isolated from a shrimp cocktail. As motile aeromonads in general are sensitive to chilling, salt, low pH, and smoke,[96] they are unlikely to constitute a serious health hazard in lightly preserved fish products.

Histamine. Histamine may be formed in lightly preserved fish products and levels between 32 and 195 mg kg^{-1} have been found in some samples of cold-smoked salmon. This is above the maximum limit of 20 mg 100 g^{-1} allowed by EU regulation,[69] however, lightly preserved fish products are not linked by epidemiological data to scombroid toxicity. The development of amines in lightly preserved fish products may result from lactic acid bacteria, some of which are able to produce biogenic amines.[165] Also, in products like matjes herring, the formation of biogenic amines has been linked to the lactic acid microflora.[199] However, Leisner et al.[165] showed that although several carnobacteria isolates were able to produce tyramine none produced histamine.

Parasites. The European legislation requires that fish raw material used for production of matjes herring, and cold-smoked fish (wild salmon, herring, mackerel, sprat, cod, and halibut) be frozen at least 24 hours at –20°C[69] because parasites are not killed by the temperatures and salt levels used in cold smoking. However, none of 3,700 fillets of farmed Atlantic salmon from Norway and Scotland was found to be infected by anisakid larvae,[10] and the legislation therefore does not apply to reared salmon.

Spoilage

Spoilage of lightly preserved fish products is mainly caused by bacterial action. Truelstrup Hansen et al.[265] found that cold-smoked salmon with reduced bacterial count was rejected by a sensory panel due to textural changes (soft and mushy), whereas the off-odors and off-flavors (putrid, sour, bitter) associated with spoilage developed in samples with natural levels of bacteria.

During chill storage (5°–10°C), the microflora of vacuum packed lightly preserved fish products becomes dominated by lactic acid bacteria. During storage of cold-smoked herring at 10°C, lactic acid bacteria increased from 10^4 cfu g^{-1} to 10^7 cfu g^{-1} after 3 weeks and 10^8 cfu g^{-1} after 6 weeks.[181] Similar data were reported for cold-smoked salmon[34,42,264] (Figure 21-4), and sugar-salted ("gravad") fish.[165] Lactic acid bacteria were found to be the dominating flora in several samples of matjes herring[199] and in lightly salted lumpfish roe.[20] High levels of lactic acid bacteria may be present for several weeks before the product is rejected for sensory reasons, and it has even been suggested that the often long keeping time of cold-smoked fish is a consequence of the development of an antagonistic flora of lactic acid bacteria[181] because they may prevent proliferation of more potent Gram-negative spoilage bacteria.

Little is known about the composition of the lactic acid bacteria group. Hildebrandt and Erol[112] found that one-third of the lactic acid bacteria on vacuum packed cold-smoked salmon were acid-sensitive carnobacteria, and a high fraction of *Carnobacterium* spp. were also found on sugar-salted fish.[165] In contrast, Truelstrup Hansen[263] reported that *L. curvatus* was the most frequently occurring lactic acid bacteria, and neither Mauguin and Novel[184] nor Jeppesen and Huss[139] found carnobacteria to be the dominant group in lightly preserved fish products. It may appear that the product either becomes dominated by carnobacteria or by *Lactobacillus* spp.

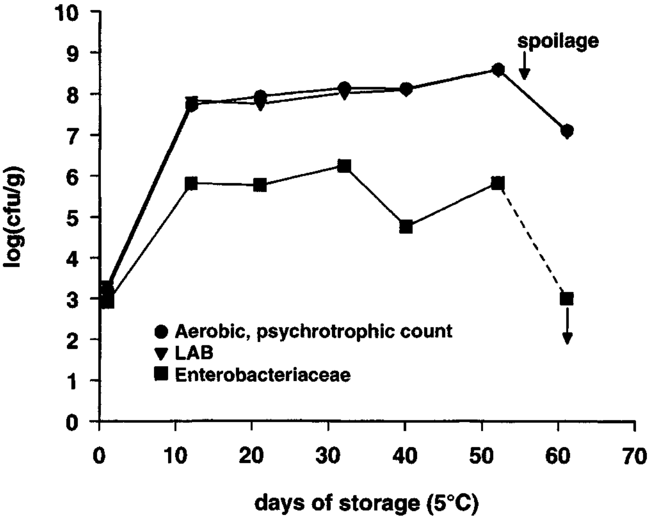

Figure 21-4 Changes in Count of Psychrotrophic, Aerobic Bacteria (●), Lactic Acid Bacteria (▼), and *Enterobacteriaceae* (■) during Storage of Vacuum Packed, Cold-Smoked Salmon (NaCl = 4.6% w/w in Water Phase) at 5°C. *Source:* Adapted with permission from L. Truelstrup Hansen, T. Gill, and H.H. Huss, Effects of Salt and Storage Temperature on Chemical, Microbiological and Sensory Changes in Cold-Smoked Salmon, *Food Research International*, Vol. 28, pp. 123–130, © 1995, Elsevier Applied Science.

Until recently, it was believed that the lactic acid bacteria were of little importance for the sensory changes during storage of fish products. Leisner[164] showed that no or very faint off-odors were produced by lactic acid bacteria compared with the very obnoxious off-odors produced by Gram-negative spoilers. However, several lactic acid bacteria were able to produce the off-odors (sour, cabbagy, sulfurous) associated with spoilage of cold-smoked salmon.[263] Furthermore, a strain of *L. saké* produced H_2S during growth on cold-smoked salmon. Counts of lactic acid bacteria in inoculated blocks of vacuum packed cold-smoked salmon reached 10^8 cfu g^{-1} after 10 days at 5°C but off-odors were not detected until after 3 weeks.[263]

Although most studies report dominance of lactic acid bacteria in vacuum packed cold-smoked fish, the chemical changes during storage vary as does the composition of the remaining microbial flora. The TMA content of cold-smoked salmon sometimes increases to 25–30 mg TMA-nitrogen 100 g^{-1},[42] whereas other samples in the same study contained only low levels (5 mg TMA-N 100 g^{-1}), and it has been concluded that TMA could not be used as an objective quality indicator of vacuum packed cold-smoked salmon.[264] The concentration of hypoxanthine increased during storage of vacuum packed cold-smoked salmon from 1–3 μmol g^{-1} to 5–7 μmol g^{-1} at rejection[34,264] and has been suggested as chemical spoilage indicator.[264]

The reason for the variability in the chemical composition of spoiled lightly preserved fish products is probably related to a yet not understood variation in the bacterial spoilage flora. Spoiled lightly preserved fish products may contain low (10^3 cfu g^{-1}) or high (10^6–10^7 cfu g^{-1}) levels of Enterobacteriaceae, *Brochothrix thermosphacta*, yeasts, and *P. phosphoreum*.[34,42,165,265] Truelstrup-Hansen[263] concluded that one of the three following different microfloras were present when cold-smoked salmon was rejected for sensory reasons: (1) lactic acid bacteria (10^7–10^9 cfu g^{-1}), (2) lactic acid bacteria and Enterobacteriaceae (10^7–10^8 cfu g^{-1}), and (3) *P. phosphoreum* (10^6–10^7 cfu g^{-1}) with occasional high levels of lactic acid bacteria. It is likely that bacterial interactions are important during spoilage of lightly preserved seafood products and Borch et al.[26] found that the spoilage profile of *Lactobacillus* spp. in cooked meat products was dependent on the presence or absence of *Hafnia alvei.*

The Enterobacteriaceae growing in lightly preserved fish products have been identified as psychrotrophic *Hafnia alvei*, *Serratia liquefaciens*, or *Enterobacter* spp. All strains produce spoilage off-odors and reduce TMAO but the ability to produce H_2S from protein substrates varies,[263] and little is known about their spoilage activity.

The sporadic occurrence of Enterobacteriaceae and *P. phosphoreum* probably explains why TMA develops in some storage trials and not in others. The complexity of the composition of the microflora and the variation seen is, apart from the sporadic occurrence of *P. phosphoreum*, similar to the microbiology of packed lightly preserved meat products.[26,50]

21.5.2 Semipreserved Seafood Products

Fish products with a high salt content (>6% NaCl in water phase) or pH below 5.0 to which preservatives (benzoate, sorbate, nitrate) may be added are defined as semipreserved. Typically, the European products (eg, salted and/or marinated herring, anchovies, caviar) are distributed at chill temperatures (<10°C). In these products, the raw material is not heat treated and destruction of parasites, and prevention of growth of microorganisms, relies on combinations of temperature, NaCl, and, for some, lowering of pH.

Several kinds of products from North Europe, particularly Scandinavia, are semipreserved. The raw material for marinated herring, the traditional barrel-salted herring is also a semipreserved product, where whole or partially gutted herring are placed in salt or sugar-salt mixtures (reaching approx. 20% NaCl wt/water phase). The herring are allowed to ripen at chill temperatures for several months and enzymes from the intestinal tract and the muscle are responsible for the ripening.[198] Usually, the ripened herring are filleted and placed in a marinade with acetic acid, salt, and sugar. Herring fillets that have been held in 4% to 5% acetic acid and 12% to 15% NaCl for 2 to 4 weeks may also be used as raw material for the marinading.[153] Also the raw material for caviar production is a semipreserved product and lumpfish roe is barrel salted (Table 21-13). The most typical semipreserved product of Southern Europe is anchovies (Table 21-13), which are produced in a

Table 21-13 Examples of European and Southeast Asian Semipreserved Fish Products

	European Products				*Southeast Asian Products*		
NaCl Level	*Addition of Sugar*	*Addition of Acid*	*Examples*	*Storage Temperature*	*Addition of CHO*[a]	*Examples*	*Storage Temperature*
5% to 10%	–	+	caviar	≤10°C	–		
	+	+	marinated fish	≤5°C	+	fermented fish	ambient or chilled
10% to 25%	–		dry-salted fish	ambient	–	fish sauces	ambient
	–		barrel-salted roe	4°–6°C	–	fish paste	ambient
	–/+		barrel-salted fish	4°–6°C			
	–		anchovies	≤10°C			

[a] CHO: carbohydrate usually added as complex carbohydrates, eg, cooked rice, cassava, or maize.

manner somewhat similar to the barrel-salted herring. Traditional ripened anchovies are made from partially gutted (or nongutted) anchovies that are placed in salt and brine and left in cans or other containers to ripen over several months.[226] The major cause of ripening is probably also intestinal enzymes.[208] As for the barrel-salted fish, aerobic nonhalophilic counts decrease during storage (to approx. 10^2 g^{-1}), and moderate growth of halophilic organisms (up to 10^4–10^5 cfu g^{-1}) is seen.[208,226]

The Southeast Asian semipreserved ("fermented") fish products may be divided into two groups: (1) one in which 20% to 30% salt is added and the fish is allowed to solubilize at tropical ambient temperature and (2) one in which lower levels of salt are combined with a carbohydrate source and a lactic acid fermentation takes place (Table 21-13).[46] Although the former group is often included in descriptions of fermented fish products, the level of bacteria (including halophiles) is very low (10^3–10^5 cfu g^{-1}).[133] The sensory characteristics of the product are probably a consequence of action of autolytic proteases rather than bacterial action. The lactic acid fermented fish products in which the salt content is lower and carbohydrates are added are receiving increasing attention.[15,46] Careful adjustment of NaCl concentration, carbohydrate source, and lactic acid starter culture is required to obtain a rapid decrease in pH,[15] and safety and sensory quality of these products is usually a close race between drop in pH and growth of pathogenic and spoilage microorganisms. Within 1 to 2 days, pH decreases to 4.5 and counts of lactic acid bacteria increase to approx. 10^9 g^{-1}.[278]

Health Hazards

Most of these products rarely, if processed and stored correctly, constitute a serious health risk as the high salt content (in some products combined with chill temperature) does not allow pathogenic bacteria to grow.[121] However, many of the reported incidences of botulism caused by fish products are due to improperly processed, semipreserved fish, often involving faulty salting or fermentation or storage at abuse temperatures.[29,186] This includes the use of poor quality raw material in which growth and toxin production of pathogens has already taken place.

C. botulinum may constitute a serious risk in semipreserved fish products. In the European semipreserved products, the risk from psychrotrophic *C. botulinum* is mainly related to usage of poor raw material as the toxin is stable in acid and salt environment (see below). The risk from mesophilic *C. botulinum* is typically caused by storage at abuse temperatures. This is unfortunately often seen for products like caviar, which contain less than 10% NaCl and must be distributed and stored at temperatures ≤10°C. However, as caviar is often packed in cans, they are sometimes placed at ambient temperature by supermarkets or consumers and this allows proteolytic types of *C. botulinum* to grow.

Fermented fish products where the salt content is lower than 10% to 12% also constitutes a serious *C. botulinum* risk. As fermentation is often carried out at ambient temperatures spores of *C. botulinum* may grow and produce toxin before the pH reaches inhibitory levels. *C. botulinum* is inhibited by pH values of 5.0 (nonproteolytic strains) and 4.6 (proteolytic strains). Fish has a high buffering capacity, but if fish is mixed with glucose (4%) and 1% NaCl, the pH drops to approximately 5–5.2 after 1 day and to 4.5 after 2 days.[267] Mixing of the fish with starchy products (eg, cassava) dilutes the buffering capacity and pH drops much more rapidly to below 4.5 within 1 day.[267] The botulinum toxin is stable in acid (pH 4.0–4.5) and salt (up to 26%) conditions[125] (Chapter 41) and once formed, further fermentation will not destroy the toxin. If the product is eaten without cooking, the risk of botulism is high. It is evident from the numerous records of botulism from various fermented fish products that this is a true high-risk group, as reviewed by Eklund[71] and Huss.[120]

Some of the risks associated with fresh fish are also present in these products, eg, marine toxins may occur also in the semipreserved product if present in the raw material. Salted anchovies have caused histamine outbreaks in the UK. However, this is believed to have been the result of formation of histamine in the raw material rather than taking place during production and ripening of the product.[226] The content of biogenic amines did not change during ripening or storage of anchovies, whereas significant production was seen after 1 to 2 days in the fresh raw material if stored at temperatures ≥8°C.[272] Most of the Gram-negative pathogenic organisms will be efficiently inhibited by the levels of salt and pH and are therefore not major problems in these types of fish products.

Nitrosamines. Nitrate is added as a preservative and coloring agent during barrel salting of some Scandinavian products, and the detection of carcinogenic nitrosamines in the product has raised concern about this addition. It has, however, also been found that the formation of nitrosamines is limited and independent of the addition of nitrate.[155]

Parasites. Fish used in pickled (marinated) products or fermented products do not receive a heat treatment necessary for destruction of parasites and as these may be present, although only in small numbers, manufacturing must guarantee their elimination. The organisms are sensitive to NaCl. Thus, Karl et al.[147] showed that in herring with 2.6% acetic acid, *Anisakis simplex* remained viable 5 to 6 weeks in 8% to 9% NaCl (in water phase) and for 10 to 12 weeks at 5% to 6% NaCl (Figure 21-5). The authors concluded that following traditional Danish procedures (presalting in 10% to 18% brine for 24 hours; brining in 10% salt and 5% acetic acid at 5°C) destroys the parasites in 5 weeks.[147]

Herring infected with the intracellular fish parasite, *Ichthyophonus hoferi,* are of poor quality (soft, slimy, and off-odorous). However, the parasite cannot grow at temperatures ≥30°C.[247] It does not infect mice and is therefore unlikely to cause disease in humans.[246]

Spoilage

The microflora of the salt-pickled European fish products have only been studied sporadically, often with the purpose of investigating the possible role of bacteria in the ripening process. Several studies have concluded that microorganisms are not important for the ripening of sugar-salted fish.[154] Similarly only low levels of bacteria ($<10^3$ cfu g^{-1}) were found in salted anchovies.[226] Knøchel and Huss[154] found that sugar-salted barrel herring could develop two spoilage patterns; one where yeast at levels of 10^5 cfu g^{-1} dominated and caused a fruity type of spoilage and one characterized by sour or putrid off-odors in which strictly anaerobic, halophilic, Gram-negative rod-shaped bacteria dominated (10^6–10^7 cfu g^{-1}). The latter was typically seen when raw material of poor quality was used because the level of TMAO was low in these fish and subsequently, the redox potential dropped more rapidly and allowed growth of true anaerobic organisms.

More heavily salted, dried fish products (eg, prepared from cod) contain 19% to 20% NaCl and 50% to 55% water. Two types of microbial spoilage can occur in these products. Halophilic bacteria (*Halococcus* spp.) that are often pigmented cause the condition known as "pink" which, apart from the formation of a reddish color in the advanced stages, causes putrid

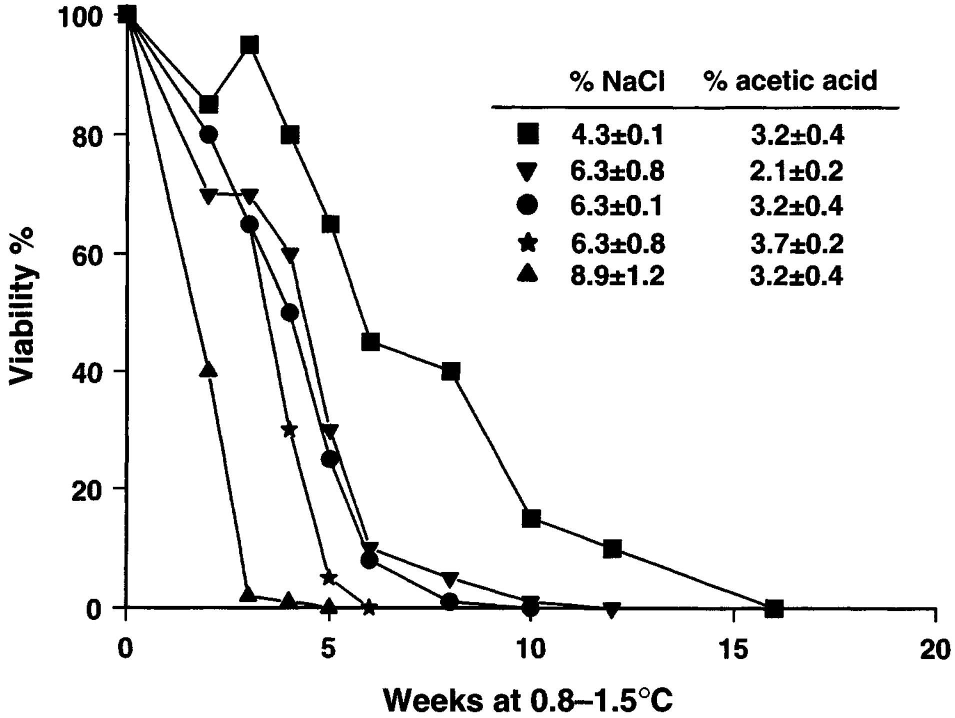

Figure 21-5 Survival of *Anisakis* Larvae in Marinated Herring Depending on Salt and Acetic Acid Concentration (% in water phase of fish tissue). Larvae were inserted (10 per butterfly fillet) in herring stored in barrels at 3°C. *Source:* Adapted with permission from H. Karl, A. Roepstorff, H.H. Huss, and B. Bloemsma, Survival of *Anisakis* Larvae in Marinated Herring Fillets, *International Journal of Food Science and Technology,* Vol. 29, pp. 661–670, © 1995, Blackwell Science, Ltd.

odors due to proteolysis.[126] Yellow, brown, and black colored molds may grow on the product causing the spoilage condition known as "dun."[126]

Little is known about the spoilage of fermented fish products; however, preliminary studies indicate that the lactic acid bacteria continue to dominate the product during storage. It is not known if spoilage of these products is caused by the activity of the lactic acid microflora or by autolytic changes.

21.5.3 Heat-Treated Seafood Products

Many seafood products receive a heat treatment as part of the processing and, depending on the temperature used, they can be classified into the following four categories with different microbiological problems:

1. Pasteurized products that are often vacuum packed and must be distributed at chill temperatures (including hot-smoked fish and Refrigerated Processed Foods with Extended Durability (REPFEDs))
2. Cooked products, eg, shrimp or cooked and breaded fish fillets, often packed and sold frozen
3. Canned (not sterilized) products
4. Commercially sterilized (canned) products

Hot-smoked fish are usually brined or dry salted and dried before smoking at an internal temperature of approximately 65°C for 45 minutes. In developing countries, the hot smoking may be a more severe process resulting in complete cooking of the meat or in a smoke-dried product. In contrast to cold-smoked fish, the proteins in hot-smoked fish are denatured.

Recently, precooked/pasteurized refrigerated foods have become very popular, particularly in catering. These products, which are not preserved by conventional chemical preservation parameters, are marketed as chill-stored products usually under vacuum or modified atmosphere and are collectively known as REPFEDs.

Pasteurized (1 minute at 77.2°–98.8°C) crab meat is typical of canned, nonsterile fish products which, as the REPFEDs, must be distributed at chill temperatures. Canned, sterilized products (eg, canned mackerel, tuna, salmon) have received sufficient heat treatment to allow distribution and storage at ambient temperature.

Health Hazards

Spores of *C. botulinum* readily survive the hot-smoking procedure and numerous studies have shown that growth and toxin production occur in the product.[71,129] Factors of importance for the time to toxin production are temperature, oxygen tension (eg, vacuum packing), salt concentration, and associated microflora. Huss et al.[129] showed that one of duplicate packs of aerobically packed hot-smoked herring was toxic after 6 days storage at 15°C, while both packs were toxic by day 16 (inoculum 10^3 spores of *C. botulinum* type E g^{-1}). If vacuum packed, both duplicate packs were toxic after 5 days at 15°C. Similarly hot-smoked whitefish with 2.8% w/v salt was not toxic after 7 days at 25°C if packed in O_2-permeable film but was toxic after 3 days if packed in O_2-impermeable film (inoculum 10^3 spores of *C. botulinum* type E 100 g^{-1}).[71] While 1.8% NaCl (w/v) was insufficient to prevent toxin production at 25°C, 3.8% prevented toxin production for 42 days at 10°C,[71] and Cann and Taylor[32] found that 3.0% NaCl prevented toxin formation for 30 days in hot-smoked trout inoculated with 10^2 spores *C. botulinum* type E g^{-1} and stored at 5°C. In hot-smoked herring that was surface inoculated with 10^3 spoilage bacteria g^{-1} and also inoculated with *C. botulinum* (10^3 spores g^{-1}), and packed in air or vacuum, toxin production occurred at the same time in both types of packs.[129] This indicated that consumption of oxygen by the associated microflora may enable growth and toxin production by *C. botulinum*. If the product is distributed at chill temperatures (≤5°C), 3% w/v NaCl is sufficient to prevent toxicity; however, at higher temperatures, more salt needs to be included. As for the lightly preserved fish products, a level of 3% NaCl combined with chill storage is necessary to prevent botulism from occurring.

L. monocytogenes is more heat resistant than Gram-negative spoilage bacteria; however, hot smoking (60°C for 20 minutes) destroyed 10^6 cells g^{-1},[136] and any risks associated with this organism in hot-smoked fish are therefore related to post-process contamination. Hot-smoked fish are virtually surface sterilized and contamination with microorganism from food handlers (eg, *S. aureus* or Enterobacteriaceae) may constitute a risk.

If the products are packed before heat treatment, post-process contamination with pathogens should obviously not occur provided that packs are properly sealed and handled, and health hazards therefore mainly concern organisms able to survive the heat treatment. The organisms of major concern in *sous vide* and REPFED foods are nonproteolytic *C. botulinum*, psychrotrophic *Bacillus cereus*, and *L. monocytogenes* as they may survive a mild heat treatment and can grow at the chill storage temperature. As many psychrotrophic Gram-negative spoilage organisms will be destroyed by the heat treatment, there is no guarantee that the product will spoil before it becomes hazardous.[5]

Due to their ability to form spores and grow at chill temperatures, nonproteolytic *C. botulinum* are of great safety concern to the REPFED industry.[178] As for *L. monocytogenes*, the composition of the substrate may strongly influence the heat resistance of *C. botulinum*, thus a $D_{82.2}$-value of 6.6 minutes was found for spores of nonproteolytic *C. botulinum* in tuna in oil, whereas oil-free fish products had D-values between 1 and 3 minutes. The subsequent chill storage period of REPFED may allow recovery of damaged cells, and studies should therefore be conducted combining heat treatment and chill storage. During experiments in which spores of nonproteolytic *C. botulinum* in a meat medium (10^6 spores 20 ml^{-1} medium) were heated at 65°C for 364 minutes, 70°C for 8 minutes, or 75°C for 27 minutes after which the medium was stored at 6°C, growth and formation of toxin occurred in all the samples within 28 days.[206] Similar experiments have not yet been conducted in fish products, and it is not known if the presence of TMAO and the positive redox potential may constitute an extra hurdle against growth of *C. botulinum*. Lysozyme is known to enhance recovery of heat-injured spores of nonproteolytic *C. botulinum*,[93,179] and because several fish contain lysozyme,[194] this may add to the risk due to *C. botulinum* in heat-treated, chilled fish products. Different countries have different recommendations for the necessary heat treatment depending on whether packed before or after heat treatment processing and depending on shelf life. However, with up to 6 weeks shelf life a heat treatment of 90°C for 10 minutes is recommended for destruction of nonproteolytic *C. botulinum*.[178]

Another potential, but not investigated, risk in REPFEDs is the growth of psychrotrophic *Bacillus cereus* (Chapter 39). In recent years, the dairy industry has paid attention to these organisms. Of 85 strains isolated from dairy products, 5 were capable of growing at 6°C, and produced toxin at 30°C.[104] Rusul and Yaakob[229] found that 164 of 187 isolates from many different foods were psychrotrophic and 82 of these were able to produce enterotoxin. In both these studies, enterotoxin production was assessed at 30° to 32°C and bears little relevance to these food products. Christiansson et al.[41] found that three-fourths of 102 psychrotrophic *B. cereus* were able to produce toxin in aerated samples of milk stored at 8°C for 72 hours, but not in nonaerated samples. The numbers of psychrotrophic *B. cereus* in foods and their growth characteristics are not known. *Bacillus* spp. grew in chill-stored *sous vide* fish, perhaps being involved in spoilage[22] (see below). This indicates that there may be a risk.

L. monocytogenes will normally be destroyed by pasteurization processes employed in the production of *sous vide* foods and other REPFEDs.[21] However, the heat resistance of the organism may vary depending on fat content and water activity; Ben Embarek and Huss[24] found that the D_{60}-value was much higher in salmon (4.5 minutes) than in cod (1.8 minutes), indicating that the lipid content of the former protected the bacteria against heat. Similar D-values have been reported for *L. monocytogenes* in various crustaceans.[21]

Cooked crustaceans, and dishes made from them, have often been implicated in foodborne diseases by *Staphylococcus aureus* and enteropathogenic Enterobacteriaceae and *Vibrionaceae*.[29,121] Typically, the heating process causes a dramatic reduction of normal Gram-negative flora and post-process contamination and some degree of temperature abuse is the usual cause of illness. The processing of several products from crustaceans involve cooking, followed by machine or manual shell removal. The latter step carries a great risk of contamination with microorganisms of human origin such as *S. auerus*. Crab that was hand picked typically contained three to four times higher levels of *S. aureus* than machine-picked meat[43]; actual levels varied from 10^2 to 10^4 g^{-1}.[43] As the product immediately after cooking is virtually sterile, the organism will grow well unless it is rapidly cooled and stored under chilled or frozen conditions.

Many of the risks described for REPFEDs also apply to the canned, nonsterile products. However, products like canned crab meat have never been the cause of botulism,[43] although it is documented that nonproteolytic *C. botulinum* may grow and produce toxin. After heating to 82.2°C for 5 minutes, Type E toxin was detected after 30 to 40 days in crab extract stored at 10°C but not detected in 75 days in crab extract stored at 4.4°C (initial inoculum 5×10^4 spores $tube^{-1}$).[166] Both storage temperature and pasteurization temperature are important parameters in controlling the botulinum hazard.

In general, it must be recommended that all fish products that are not heat treated immediately prior to consumption (ready-to-eat products), and that rely on chill temperature as the only hurdle against *C. botulinum*, should contain a minimum of 3% NaCl wt/water phase.

Canned, sterilized products pose no health risk if processed (heat treated) correctly using good quality raw materials. However, canned fish products have been implicated in several cases of histamine poisoning[260] resulting from histamine accumulated in the raw fish before thermal processing.[1] The heat processing of low-acid canned foods is specifically designed to eliminate *C. botulinum* and canned fish account for only 3% of outbreaks of botulism.[120] Botulism from canned products is caused by underprocessing and/or post-process contamination. Thus, two out of four commercially prepared fish products that have caused botulism involved post-process contamination of canned salmon.[186]

Spoilage

Hot smoking of fish will reduce the bacterial load and if the product is vacuum packed and stored at 5°C the bacterial count remains low ($<10^3$ g^{-1}) even after months of storage.[85] The quality, however, decreases as a dry unpleasant texture and flavor appears. If stored aerobically, molds and yeasts are common spoilage organisms.[85] In many African countries, hot smoking and smoke drying are widely used as means of preservation. The products are typically not salted and stored aerobically at ambient tropical temperatures. Molds of various kinds develop on the surface and are typically the cause of rejection,[70] together with insect infestation.

Little is known about the spoilage of REPFEDs; however, in products stored in chilled conditions, psychrotrophic, heat-resistant microorganisms would be expected as the main cause of microbiological spoilage. The microbial count of *sous vide* cod stored at 5°C sporadically increased to 10^6–10^8 cfu g^{-1} and extremely obnoxious and putrid off-odors developed after 4 to 5 weeks.[22] Gram-positive, motile, aerobic bacteria with oval and terminal spores were isolated and identified as a *Bacillus* species.[22]

Unusual clostridia have been found as spoilers of pasteurized crab meat,[237] and it has been reported that gasses like CO_2 or a combination of CO_2 and H_2S were produced in spoiled pasteurized crab meat in which the facultative anaerobic count was approximately 10^8 g^{-1}.[43]

Properly processed canned foods present no microbiological problems and container defects and underprocessing were almost always involved when canned products spoiled.[2]

21.6 CONCLUDING REMARKS

Many aspects of the microbiology of fish and fish products have been thoroughly investigated, and as is evident from the above, the understanding of both health hazards and spoilage is, for some products, well established. For instance, the microbiology of products that are heavily preserved (eg, iced, high salted, heat sterilized) is well understood. However, it is also clear that there are many areas that require further investigation, partly because changing, often milder processing procedures are increasingly used. This is particularly so for products in which combinations of mild preservation parameters are used.

In general, research should be focused on understanding the ecology of the microorganisms in their environment. This will, as opposed to much of the research done so far, require studies in food-relevant model systems and studies of mixed cultures. An attempt should be made to control the development of the microflora in a product, using the nonpathogenic microflora, rather than focusing on complete elimination or inhibition of the microflora.

Some of the major areas needing further investigation are:

1. the ecology of viral contaminants
2. a fuller understanding of the ecology of pathogenic bacteria associated with fish and fish products, eg, how do the organisms cope with transfer from nutrient rich (foods and humans) to nutrient poor conditions such as soil and water
3. contamination routes, eg, for *L. monocytogenes*
4. the development of better control measures for *L. monocytogenes* and other psychrotrophic pathogens
5. identification and characterization of specific spoilage bacteria in fish products, particularly the lightly preserved fish products
6. characterization of the microbiology of fermented fish products
7. the importance of bacterial interactions for growth and metabolism of spoilage and pathogenic organisms
8. risk assessment of fish products
9. assessment of advantages and risks of new preservation principles
10. the possible consequences of widespread use of antibiotics in fish rearing.

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22 Milk and Unfermented Milk Products

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22.1 INTRODUCTION

Milk and products derived from milk have been part of the human diet throughout recorded history. Despite current controversies surrounding the nutritional status of milk, it remains an important part of our diet and is a valuable source of calcium, protein, and vitamins. The dairy industry must ensure that the dairy products we consume are of the highest quality and are safe to eat. To accomplish this a good understanding of the microbiology of milk must be maintained, even though the problems may change with the introduction of new production and processing systems. This chapter will review the current status of knowledge concerning the microbiology of milk and milk products from farm to carton, and point out some of the concerns that may arise in the future. Before microbiological issues are addressed, a brief overview of the composition of milk is provided as it is impossible to understand factors affecting the growth and survival of organisms without knowledge of the substrate availability and the food environment in which the organism is found.

22.2 MILK COMPOSITION

22.2.1 What Is Milk?

Milk is a complex source of nutrients including protein, carbohydrate, lipid, vitamins, and minerals whose primary role is to provide nourishment to the neonates of the mammalian species from which it was derived. However, milk from a variety of animals has become an important part of the human diet. The components that make it nutritious for humans also provide an ideal growth medium for many microorganisms. Although the composition varies significantly with species and breed, the typical composition of bovine milk is shown in Table 22-1.

22.2.2 Lipids

About 98% of the fat of cow's milk is triglyceride, the rest is composed of small amounts of mono- and diglycerides, phospholipid, free fatty acids, cholesterol ester, and cholesterol. The lipid contains mainly C_{16} to C_{18} fatty acids but a significant amount (about 38%) of the triglyceride contains short-chain C_4 to C_{14} fatty acids.[42] The fat in milk is present as globules surrounded by a phospholipid-rich membrane (the milk fat globule membrane).[177] Homogenization, or other mechanical stress, causes a reduction in size of the globule and makes the lipid more accessible to the action of lipolytic enzymes.

Several of the microorganisms isolated from milk are capable of hydrolyzing milk fat. In a survey of creamery silo milks in southwest Scotland, Muir et al.[176] found that the majority of isolates obtained were capable of lipolytic activity (Table 22-2). These included members of the genus *Pseudomonas* as well as Enterobacteriaceae. It is well recognized that several psychrotrophic bacteria produce heat-resistant lipolytic enzymes that can remain active even after pasteurization.[103,141,231] These enzymes also retain significant activity at refrigeration temperatures.[5,40,60,85,194,235] In the presence of other enzymes, such as protease, glycosidases, and phospholipase, whose combined effect is to break down the milk fat globule membrane, the action of lipase is enhanced.[3,39,93,153,187] It is believed that the milk fat globule membrane does not limit the activity of bacterial lipases.[84] This view is supported by results showing that only the native milk lipoprotein lipase and not *Pseudomonas fluorescens* lipase exhibited the synergy with other enzymes responsible for degrading the fat globule membrane.[93] It has been shown that *Staphylococcus aureus* can bind to milk fat globules and this results in a "biofilm-type" of growth in which the bacterial cells form a capsule.[2] The staphylococcal cells bound to the fat globule are more resistant to antibiotics than planktonic cells. It may be that several other bacteria may be able to associate with fat globules and exhibit capsule formation, which would make them more resistant to sanitizers as well as antibiotics.

The lipid composition of milk can be varied by dietary manipulation.[9] When cream was obtained from milk with high (37.2%) and low (27.8%) levels of unsaturated fatty acids, the shelf life of the cream high in unsaturates was 2.5 days longer at 8°C.[194]

22.2.3 Proteins

The major proteins found in milk are the caseins and the whey proteins comprising α-lactalbumins, β-lactoglobulins, serum albumin, and the immunoglobulins.[10] Psychrotrophic bacteria found in milk produce heat-stable extracellular proteases that primarily act on the caseins. These are degraded usually in the order κ-casein > β-casein > α-casein.[72] However, other degradation patterns have been described.[238] Proteolysis of milk usually results in gelation[72,159] and in the production of bitter and "unclean" off-flavors.[156,262] The psychrotrophic microflora found in milk do not generally degrade whey proteins; however, the lactic acid bacteria are known to denature these proteins. Proteins that can exert a considerable effect on the microflora of milk are present at low concentrations and these include lactoperoxidase, lactoferrin, and lysozyme.

22.2.4 Carbohydrates

Lactose is the principal carbohydrate found in milk, although monosaccharides (primarily glucose and galactose), oligosaccharides, and peptide- and protein-bound carbohydrates are also present, albeit at very low concentrations. Lactose is the ferment-

Table 22-1 Typical Composition of Bovine Milk

Proximate Composition		*Protein Composition*		*Nonprotein Nitrogen*		*Trace Elements*		*Vitamins*	
Constituent	*Conc. (g liter^{-1})*	*Constituent*	*Conc. (g liter^{-1})*	*Constituent*	*Conc. (mg N liter^{-1})*	*Constituent*	*Conc. (μg liter^{-1})*	*Constituent*	*Conc. (mg liter^{-1})*
Fat	37	Casein	26	Ammonia	9	Aluminum	50–2100	Water-soluble	
Protein	32	α_{s1}	10.4	α-amino acids	44	Arsenic	10–400	Thiamine	0.44
Lactose	48	α_{s2}	2.6	Hippuric acid	4	Boron	30–800	Riboflavin	1.75
Non-protein N	0.19	β	9.1	Urea	142	Cobalt	0–20	Nicotinic acid	0.94
Calcium	1.25	γ	1.3	Creatine	25	Iron	100–2400	Pyridoxine	0.64
Phosphorus	0.96	κ	2.6	Creatinine	12	Manganese	3–370	Pantothenic acid	3.46
Magnesium	0.12	α-lactalbumin	0.7	Uric acid	8	Molybdenum	5–150	Biotin	0.031
Sodium	0.58	β-lactoglobulin	3	Orotic acid	15	Silicon	1300–7000	Folic acid	0.05
Potassium	1.38	Serum albumin	0.3	Carnitine	1.2	Zinc	220–19000	B12	0.0043
Chloride	1.03	Immunoglobulin	0.6	Acetyl-carnitine	0.8	Bromine	60–25000	Choline	121
Sulfur	0.3	Lactoferrin	>0.018	Phosphoryl-ethanolamine	8.3	Iodine	5–700	Inositol	50
Citric acid	1.75							Ascorbic acid	21.1
CO_2	0.2			Glycero-phosphoryl-ethanolamine	2.9			Fat-soluble A	0.4
				N-acetyl-glucosamine	7				
				N-acetyl-glucosamine-1-phosphate	7.3				

Source: Data from W. Banks and D.G. Dalgleish, Milk and Milk Processing, in *Dairy Microbiology Volume 1: The Microbiology of Milk,* 2nd ed., R.K. Robinson, ed., pp. 1–35, © 1990, Elsevier Applied Science, and P. Walstra and R. Jenness, *Dairy Chemistry and Physics,* © 1984, Wiley-Interscience.

Table 22-2 Types and Associated Enzymatic Activity of Bacteria Isolated from Creamery Silo Milk

Genus	*% Isolates in Genus*	*% Proteolytic Isolates in Genus*	*% Lipolytic Isolates in Genus*	*% Isolates in Genus with Both Enzymic Activities*
Pseudomonas	71.2	3	37	82
Enterobacteriaceae/*Aeromonas*/*Pasteurella* or *Vibrio*	7.7	2	2	31
Acinetobacter/*Moraxella* or *Brucella*	5.6	9	5	36
Chromobacterium	2	6	25	41
Flavobacterium	3.6	6	6	24
Alcaligenes	1.4	0	0	92
Gram-positives	6.9	2	10	12
Unclassified	2.7	0	6	25

Source: Data from D.D. Muir, J.D. Phillips, and D.G. Dalgleish, The Lipolytic and Proteolytic Activity of Bacteria Isolated from Blended Raw Milk, *Journal of the Society of Dairy Technology*, Vol. 32, pp. 19–23, © 1979.

able carbohydrate utilized by the lactic acid bacteria during the production of cheese, yogurt, etc. Methods of transportation and metabolism of lactose vary substantially within the lactic acid bacteria.[44] Certain lactic acid bacteria can also metabolize citric acid present in milk to produce flavor compounds.

22.2.5 Vitamins and Minerals

Milk also provides a good source of vitamins and minerals for microbial growth. Thus, an ample nutrient supply coupled with a near neutral pH (6.5 to 6.8) and a high water activity ensures that milk will support the growth of a wide range of microorganisms and, as such, is a highly perishable product. The need to preserve milk has led to the development of a variety of products that rely on temperature, a reduction in water activity, or both to maintain stability.

22.3 UNFERMENTED PRODUCTS MADE FROM MILK

22.3.1 Fluid Milk

Pasteurized Milk

Milk for consumption is generally heat treated to reduce numbers of potential pathogens and, concomitantly, spoilage organisms. This is achieved by passage through a plate heat exchanger at high temperatures for short holding times (HTST pasteurization). The time-temperature combinations used vary considerably but the legal minimum requirement in most countries is at least 72°C for not less than 16 seconds.

UHT Milk

In order to create a shelf-stable product that can be stored at ambient temperatures for several months, commercial sterility is required. To achieve this without having catastrophic effects on flavor, milk is heated at very high temperatures (≥125°C) for very short holding times (1 to 5 seconds). This ultrahigh temperature (UHT) treatment can be achieved either indirectly by using a heat exchanger or by direct injection of steam into the milk. The latter is claimed to have advantages in terms of taste. No matter how the heat treatment is carried out, to maintain sterility the milk must be aseptically packaged.

22.3.2 Cream

The fat may be separated from milk by a continuous centrifugation process, which makes use of the density differences between the fat and the aqueous phase. Creams of different fat content are produced and their composition varies among countries. Typically, the fat content can vary between 12% and 55%. Creams of lower fat content are almost always homogenized. As with fluid milk, cream is heat treated to ensure safety and both HTST pasteurized and UHT creams are produced. The temperatures used in the HTST process are usually higher for cream than for milk.

22.3.3 Butter

During butter manufacture cream is churned and "worked" to produce a continuous fat phase consisting of fat globules that have coalesced, fragments of globules, and fat squeezed from the globules. The major portion of the aqueous phase is present in small discrete droplets but some of the water forms continuous channels. An accepted standard for butter in most countries is that it should not contain more than 16% water and not less than 80% milk fat. To improve the spreadability of butter attention has focused on vegetable oil–butter blends. Cream for butter manufacture is pasteurized before churning. Following pasteurization, the hot cream may also be subjected to reduced pressure in a process called vacreation. This removes undesirable feed-related taints from the butter.

22.3.4 Milk Powder

The properties of the powder are determined by the processing conditions used in its manufacture. Low-heat powders are manufactured from pasteurized milk and, in this case, the integrity of the whey proteins is intact. These powders are normally used as coffee whiteners. High-heat powders are produced from milk that has undergone a second heat treatment at 115° to 120°C for 30 seconds to 15 minutes to effectively denature the whey proteins. These powders are ideal for incorporation into bread dough. Following the initial heat treatment, the milk is evaporated to about 50% total solids and then spray dried. The evaporated milk is atomized, and the small particles that result are dried very quickly by the application of heat. The heat can be applied directly or indirectly. Although the temperatures in the drier can reach 180°C, the temperature of the particles never exceeds that of their dew point and so damage to protein is minimal. The moisture content of the powder collected from the drier is about 4%. The finer particles are collected using a cyclone.

Instantized powders that are readily soluble can be obtained by modification of the drying process to allow agglomeration of particles. Powders are removed from the drier at 8% moisture during this process and final drying is achieved on a fluidized bed.

22.3.5 Evaporated and Condensed Milks

In order for the milk protein to withstand the in-can sterilization process used in the manufacture of evaporated milks, stabilizers such as phosphates, citrate, bicarbonate, or $CaCl_2$ are added to the raw milk. The milk is heated to denature the whey proteins and then evaporated. Heat-treated skim milk concentrate is added to reduce the total solids and fat content to the required levels. Following homogenization, the evaporated milk is filled into cans, sealed, and sterilized at 120°C for 10 minutes. Evaporated milk is darker in color than pasteurized milk because of the Maillard browning that occurs at the high temperatures used in the process.

Condensed milks are manufactured in a similar way to evaporated milks but they are not sterilized. Sweetened condensed milk is made by the addition of sugar (usually sucrose) to the milk. For the retail trade, where the milk is canned, a "sugar ratio" of 63.5 to 64.5 is used but for bulk sales to confectioners, bakers, and other users a ratio of 42 is more common because of the shorter anticipated shelf life. The sugar ratio is determined from the formula:

$$\begin{array}{c}\text{Sugar}\\\text{ratio}\end{array} = \frac{\%\ \text{sugar of condensed milk}}{100 - \text{total milk solids of condensed milk}} \times 100$$

22.3.6 Ice Cream

Ice cream is manufactured from a liquid mix that may contain milk, cream, water, milk solids-not-fat, nonmilk fat, sugars, emulsifiers, stabilizers, and flavoring and coloring agents. The mix is heat treated either under HTST (usually about 80°C for at least 16 seconds) or UHT conditions (usually about 140°C for 5 seconds), homogenized, cooled, and stored at about 3°C for up to 24 hours. The mix is rapidly frozen in an ice cream freezer to temperatures in the range –5° to –7°C. During freezing air is incorporated by agitation of the mix. After freezing the ice cream may be dispensed for sale as "soft-serve" or can be packaged and hardened at –20° to –40°C.

Dairy desserts are made from ingredients similar to those in ice cream mix formulations.

22.4 MICROFLORA OF RAW MILK

22.4.1 Production of Milk

Although milk production practices differ greatly throughout the world, in most developed countries milk is collected by machine milking and transferred to refrigerated bulk storage tanks where it is held prior to transportation to processing facilities. These handling methods have resulted in a dramatic change in the microflora of raw milk brought about by selection and adaptation. However, before we investigate these changes, some background information on the sources of microbial contamination of milk would be opportune.

22.4.2 Contamination from the Udder

Contamination from Udder Infection

In healthy cows free from infection, milk emerging from the udder is essentially sterile. However, udder infections are common, and there is evidence that about 33% of dairy cattle in the United Kingdom (UK) suffer from mastitis.[27] The most common agents of mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Escherichia coli*. As well as producing visible clinical infection, less acute, subclinical states are often encountered and can only be diagnosed by examination of the milk for characteristic changes, such as elevated somatic cell counts and increases in concentrations of serum albumin and immunoglobulins in the milk. The organisms enter the udder by way of the duct at the teat tip and certain of them, such as *Staphylococcus aureus*, can colonize the duct. It is thought that machine milking plays a part in the propulsion of the organisms into the teat duct but this is by no means the only route of contamination.[27] From the duct the organisms can enter the milk and can contribute significantly to the numbers present in bulk tank samples. Bramley et al.[28] isolated *S. aureus* or mastitis-producing streptococci from 86% of milk samples analyzed but bacterial numbers were below 1×10^4 cfu ml^{-1} in 90% of these milks. However, in herds with a high incidence of *Streptococcus uberis* infections, counts in the bulk tank milk reached 1×10^5 cfu ml^{-1} in a small number of cases.

Whereas the organisms that cause mastitis do not generally grow in refrigerated milk, they are able to survive under these conditions and may be a concern from a public health aspect. It has been demonstrated that staphylococcal enterotoxins and toxic shock syndrome toxin-1 can be presynthesised in the udder and secreted into milk in cows and goats suffering from *S. aureus* mastitis.[181,265] Modern dairy husbandry practices, such as teat dipping and dry cow therapy with antibiotics, can successfully reduce the incidence of mastitis.[26,114] Hillerton et al.[115] showed that application of a mastitis control plan reduced the incidence of cows infected with mastitis caused by coagulase-positive staphylococci from 21.9% to 12.0% in a 5-year period. However, the total incidence of clinical mastitis did not change significantly because environmental organisms were responsible for 65% of all clinical cases. It has also been suggested that reductions in somatic cell counts in raw milks observed in some countries may be due to a reduction in the practice of addition of mastitic milk to the bulk supply by farmers, especially where payment schemes based on milk quality are in operation.[196]

The control of mastitis through antibiotic therapy does not come without a price. As well as the concerns over potential allergenic, carcinogenic, and mutagenic reactions in consumers who are exposed to milk containing antibiotic residues, antibiotic therapy with drugs such as β-lactams and tetracycline may result in the transfer of antibiotic resistance to human pathogens from drug-resistant organisms. However, whereas some researchers believe that the incidence of the latter is negligible,[167] there is now increasing evidence that antimicrobial drug use on farms can lead to the dissemination of antibiotic resistance via the food chain.[158] Multiple antibiotic-resistant strains of a number of organisms, including *E. coli*, *Salmonella* spp., and *S. aureus*, have been isolated from raw and pasteurized milk,[57] and there are reports of a decrease in efficacy of several antibiotics against some *E. coli* that cause mastitis.[73] Their presence in pasteurized milk may have been the result of post-process contamination or inadequate heat treatment. There is another concern that has not been adequately addressed, and that is the possibility of the development of cell-wall deficient (CWD) or L-form strains of bacteria arising in the milk supply. These CWD strains arise as a result of exposure to antibiotics or other antimicrobial agents that affect the cell wall. They are often very difficult to detect by standard culture techniques although they remain viable. The role of CWD strains in a number of diseases has been reviewed by Mattman.[155] CWD strains of *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Corynebacterium pyogenes* have been implicated in bovine mastitis.[18] Sears et al.[220] found that cows infected with *S. aureus* that had been treated with cloxacillin did not secrete the classic form of the organism in their milk but CWD forms could be detected in the milk for up to 30 days. The practical significance of these findings remains to be determined.

There is evidence that significant numbers of organisms are found in milk taken aseptically from the udders of apparently healthy cows. Limond and Griffiths[147] obtained counts of about 100 cfu ml^{-1} in aseptically drawn milk, although no attempt was made to characterize these isolates. Apart from mastitis-causing organisms, other bacteria that are pathogenic to humans may infect the udder; these include *Mycobacterium bovis*, *Brucella abortus*, *Listeria monocytogenes*, *Coxiella burnetii*, and *Salmonella* spp.[259] Recent attention has focused on *Mycobacterium paratuberculosis*, which has been linked to Crohn's disease.[37]

Contamination from the External Surface of the Udder

The external surface of the udder is also a prime source of microbial contamination of milk. Bedding materials, mud, dung, soil, and other matter all readily stick to skin and are a rich source of microorganisms. Even after washing with water, the microbial count on teat surfaces can be high,[250] and the count in milk from washed udders may only be about 1 log cycle lower than from those that were unwashed.[126] However, the importance of proper washing and drying of the udder before milking for the elimination of *Listeria* spp. has been demonstrated.[121] In a study on the risk factors associated with contamination of raw milk by *Listeria monocytogenes* on dairy farms, Sanaa et al.[213] showed that poor cleanliness of cows, inadequate lighting of milking parlors and barns (which may be an indication of neglect of milking hygiene), and incorrect disinfection of towels used to dry the udder significantly increased the likelihood of contamination.

It has been suggested that bedding affords the greatest contribution to udder contamination.[49,161,225] These researchers showed that there was a reduction in bacterial levels on teats when cows were on pasture, and this was reflected in lower bacterial counts in milk during this period. The bacterial count of all types of bedding was about 5×10^9 cfu g^{-1} whereas that on pasture was approximately 8×10^7 cfu g^{-1}.[161]

The dominant microflora on the teats of cows housed in byres were micrococci,[48,49,257] but it has also been estimated that 90% of the spores found in raw milk come from this source.[161,264] The principal source of psychrotrophic spore formers (mainly *Bacillus* spp.) in milk appears to be contamination of the teat by the upper layer of soil in pasture land and by feces[161,247] but this obviously does not hold for cows that are zero-grazed. There is also a distinct seasonal effect on the incidence of psychrotrophic spore formers in milk, with the highest levels being observed in late summer and early autumn.[161,193,241] McKinnon and Pettipher[161] determined that the mean total spore count in farm bulk tank milks during the winter was 2.2×10^2 cfu ml^{-1} whereas the corresponding value in the summer was 10 cfu ml^{-1}. However, the summer milks contained about 5 psychrotrophic spores ml^{-1} compared with 0.5 ml^{-1} in milks sampled in the winter. It has been postulated that antagonistic effects of mesophilic strains of *Bacillus* spp. may influence the incidence and growth of psychrotrophic isolates in the environment or in milk.[241] *Clostridium* spores can be introduced into milk from feedstuff, especially silage, and bedding.[33,59,113] Silage is also an important source of contamination by *Listeria* spp. and other potential human pathogens such as *Yersinia enterocolitica* and *Aeromonas hydrophila*.[92,213]

22.4.3 Environmental Sources of Contamination

Personnel

It is unlikely that personnel contribute significantly as a source of microbial contamination of milk during machine milking, although workers suffering from certain zoonoses, such as Q fever, should not be allowed to participate in the milking process.[27]

Aerial Contamination

Air is also an insignificant contributor to microbial contamination of raw milk. It has been calculated that airborne bacteria account for <5 cfu ml^{-1} of the bacterial load of milk; of these *Bacillus* spores would constitute <1 cfu ml^{-1}.[15,264]

Water

Water used in the production of milk should be of potable quality. Storage tanks should be protected to prevent access by insects, rodents, birds, and other sources of contamination and equipment used to deliver water should be properly cleaned. Problems may arise where untreated water supplies are used to rinse and wash equipment. Such water may contain a diverse array of microorganisms including *Pseudomonas* spp., coliforms, *Bacillus* spp., and numerous other genera.[27] The number of cells contaminating the milk may be small but there is the potential for growth in any residual water remaining on the equipment. Chlorination of the water used in the production of milk is recommended. Concerns about the use of untreated water, and even of mains supplies, have been heightened in recent years by the increased incidence of *Cryptosporidium parvum*. It is known that oocysts of this protozoan can be present in raw milk,[120,136,228] albeit at low incidence rates (<1%), but their source is undetermined. However, it is unlikely that the oocysts can survive commercial pasteurization.[109]

22.4.4 Contamination from Milking and Storage Equipment

Significant contamination of milk can arise from inadequately sanitized surfaces of milking and milk storage equipment. McKinnon et al.[162] demonstrated that the total bacteria count of milk may increase by up to 3×10^3 cfu ml^{-1} due to milking equipment and a further 1.5×10^3 cfu ml^{-1} from the bulk tank. The organisms proliferate in milk residues present in crevices, joints, rubber gaskets, and dead-ends of poorly cleaned milking plants.[252,256] A diversity of bacterial types can be introduced into milk from milk mineral deposits present in milking equipment.[251-256] Arguably, the most important of these are the Gram-negative psychrotrophs, which predominate among the microflora that adhere to stainless steel milk transfer pipelines.[146]

Differences in cleaning regimes and, hence, the level of contamination from farm to farm ensure that considerable variation occurs in the microflora of milking equipment.[61] The only real protection against the introduction of bacteria into the milk supply during milking is adequate sanitation of all the equipment.[32,83] Variations in temperature and cleaning procedures affect the attachment of bacteria to stainless steel surfaces.[34,236] The effectiveness of sanitation depends to a large extent on the design of the plant and on other factors such as the hardness of the water supply, which itself can give rise to deposits on milking equipment.[188]

Farm bulk tanks do not contribute greatly to the bacterial load of raw milk because they are easy to clean and, consequently, have a much lower bacterial content than the milk pipeline.[61] However, ancillary equipment such as agitators, dipsticks, outlet plugs, and cocks can be difficult to clean and may be a possible source of contamination.[27] There is also some speculation that inadequately cleaned bulk tanks can be a source of psychrotrophic spore formers.[163] But by far the most important contribution to microbial load afforded by bulk tanks is potential growth of contaminants during storage.[194] Economics is dictating that milk be collected from farms on alternate days, or even longer in some instances. Thus, at collection part of the milk in the bulk tank may be 48 hours old or more. Whereas most of the surveys conducted indicate that alternate day collection has little effect on the bacteriological quality of bulk tank milk providing it was cooled rapidly to 4°C or below before addition to the tank,[61] the "growth potential" of the raw milk microflora is significantly affected.

The length of the lag phase of growth of the native microflora in farm bulk tank milk stored at 2° to 4°C was about 48 hours[105,175] (Figure 22-1). These data support those of Bockelmann[23] who suggested that the critical time for storage of milk at refrigeration temperatures was between 60 and 72 hours. Thus, milk from alternate day collections arriving at a processing site will contain organisms that are entering the exponential phase of growth, and the amount of time that this milk can be subsequently stored will be adversely affected.[185,186] Further evidence that prolonged cold storage can affect the spoilage potential of contaminating bacteria was obtained by Jaspe et al.[124] These workers compared *Pseudomonas* spp. isolated from milk shortly after milking with those isolated from the same milk samples that had been stored at 7°C for 3 days. The strains from the stored milk grew 10 times faster at 7°C, had 1,000-fold more proteolytic activity and were 280-fold more lipolytic than the strains isolated from the freshly drawn milk. However, during the storage period, the *Pseudomonas* counts in the milk increased by only about 1 log cycle. The microbial load prior to bulk storage can also greatly affect the length of time the milk can be safely held.[87,105] Milk produced under poor hygienic conditions will have a short storage life, even at low temperatures.

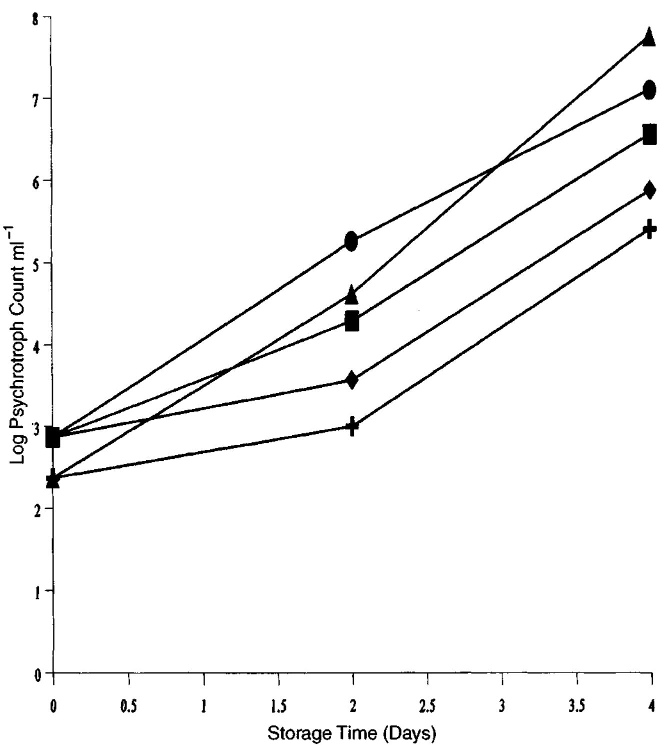

Figure 22-1 Growth of the Natural Microflora of Raw Milk at 2°C (✚; data of Ref. 105); 4°C (◆; data of Ref. 175); 6°C (■; data of Ref. 175); 6°C (▲; data of Ref. 105); and 8°C (●; data of Ref. 175). Counts were obtained on milk agar incubated at 21°C for 25 hours.

Some studies have aimed to determine the relative contributions of contaminants from the udder and from the milking plant. Kurzweil and Busse[135] determined that udder microflora were predominant in low-count milk but, as the microbial load in raw milk increased, a greater proportion of the organisms were derived from the milking equipment. This suggests that high-count milks are the result of inadequate cleaning of milking machines and associated pipelines.

22.4.5 Contamination during Transportation and Storage at the Processing Facility

Dairy equipment used for the collection, transport, and storage of refrigerated raw milk becomes fouled with a milk film, which can support growth of bacteria that consequently become a source of contamination to subsequent batches of milk. Thus, adequate cleaning must be ensured. Washing is usually performed at high temperatures (70°C) but this may exacerbate problems due to heat-induced fouling. Low temperature (45°C) wash procedures can be successfully applied to clean milk collection tankers and storage tanks.[233,234]

Contamination during Transportation

Milk is usually transported in insulated tanks or in tankers equipped with refrigerated storage. The drivers of these vehicles are usually authorized to refuse obviously abnormal milk or milk that is above a certain temperature. Milk may be transferred to larger vehicles for longer journeys. The main causes of increased bacterial count during this stage are contamination due to inadequately cleaned vehicles and growth of bacteria already present in the milk. The latter is obviously dependent on the temperature of the milk and duration of the journey. It has been estimated that a two-fold increase in count is common during transportation from the farm to the processing site because of the proliferation of psychrotrophic bacteria, including coliforms.[143,249]

Bell et al.[12] studied the efficacy of a rapid, ATP bioluminescence-based test for the evaluation of tanker cleanliness. They found that only 63% to 89% of surfaces examined were assessed as free from extraneous contamination. Externally accessible areas such as manways, hoses, and the air elimination vessels were consistently dirtier than the inside of tankers. Critical sites for cleaning have been identified as the air separator, the milk meter, the milk sieve, and the suction hose.[233,234] Factors that contribute to inadequate cleaning include blockage of the cleaning-in-place (CIP) spray system and low water pressure and flow rate. These can lead to build-up of milkstone on the inner surface of the tanker.

Contamination during Storage at the Processing Site

Changes in dairy industry practices, such as the introduction of a 5-day working week and shortages at certain times of year due to the adoption of quota systems, have led to milk being stored for longer times before processing. Because of this, the temperature at which milk is stored becomes critical. It has been recommended that the milk is cooled to, and maintained at, 3°C on receipt at the processing plant before storage.[111] Milk is usually stored in insulated silos prior to processing and can be held under these conditions for 2 to 3 days. The temperature gain in the silos should not exceed 1°C at maximum ambient temperatures over a 24-hour period.[111]

Muir et al.[174] surveyed the bacteriological quality of silo milk at several dairies in southwest Scotland and found the averages for the number of aerobic bacteria and number of psychrotrophic aerobic bacteria to be 1.7×10^5 cfu ml^{-1} and 1.3×10^5 cfu ml^{-1}, respectively. The majority of bacteria present were pseudomonads (70.2%) but Enterobacteriaceae (7.7%), Gram-positive bacteria (6.9%), and other Gram-negative, rod-shaped organisms were also isolated.[176] When the milks were stored for a further 48 hours at 6°C, the psychrotroph count increased by 2 log cycles to 1.3×10^7 cfu ml^{-1}.[175] Psychrotrophic growth patterns were similar whether milks were selected according to their initial total aerobic counts or whether they were stored in large, air-agitated silos or small, paddle-agitated vats.[58] Growth rates were highest during filling of the silos, due possibly to temperature fluctuation, and final bacterial numbers were dependent on initial counts and storage time, with the latter being the most significant factor affecting final quality. Methods to predict growth rates of psychrotrophic microorganisms present in milk have been reviewed by Griffiths.[96]

Following a study of the spoilage of raw and pasteurized milk, Ternström et al.[248] concluded that raw milk was spoiled exclusively by Gram-negative bacteria. Of these, *Pseudomonas fluorescens* biovar I (32.1% of isolates), *P. fragi* (29.6%), *P. lundensis* (19.8%), and *P. fluorescens* biovar III (17.3%) were the most commonly isolated. Other organisms isolated belonged to the Enterobacteriaceae and the genus *Acinetobacter*. *P. fluorescens* biovar I was also the most frequently isolated pseudomonad from raw milk in a study undertaken by Gennari and Dragotto.[88] There is little difference between the types of spoilage associated with bovine milk and ovine and caprine milks.[182,258] Some of the common bacteria isolated from bovine milk and their sources are shown in Table 22-3.

22.4.6 Consequences of Growth of Psychrotrophic Bacteria in Raw Milk

As well as being able to grow rapidly in refrigerated milk, psychrotrophs produce extracellular enzymes that can degrade milk components. Certain of these, proteases and lipases, were alluded to earlier in this chapter. Although the majority of psychro-

Table 22-3 Bacteria Commonly Isolated from Raw Milk

Genus	*Source*
Gram-negative	
Achromobacter (now *Alcaligenes* or *Pseudomonas*)	Soil
Acinetobacter	Soil, water, milking equipment
Aeromonas	Water
Alcaligenes	Soil, water, milking equipment
Alteromonas	Soil, water
Campylobacter	Feces
Chromobacterium	Soil, water
Citrobacter	Water, feces
Enterobacter	Mastitic infections, water, milking equipment
Escherichia	Bedding, mastitic infections, water, milking equipment
Flavobacterium	Soil
Klebsiella	Sawdust bedding, mastitic infections, water, milking equipment
Pseudomonas	Soil, streak canal of udder, water, milking equipment
Salmonella	Feces
Serratia	Soil, water, milking equipment
Yersinia	Feces
Gram-positive	
Arthrobacter	Teat surfaces
Bacillus	Bedding, pasture
Clostridium	Feces, fodder, bedding, teat surface
Corynebacterium	Streak canal of udder
Enterococcus	Feces, water
Lactobacillus	Feces, silage
Listeria	Soil, water, fodder, feces
Microbacterium	Milking equipment
Micrococcus	Teat surface, streak canal of udder, milking equipment
Sarcina	Soil, water
Staphylococcus	Teat surface, streak canal of udder
Streptococcus	Teat surface, streak canal of udder

trophs present in milk are not heat resistant,[194] in many cases the extracellular enzymes that they produce can survive pasteurization (70° to 80°C) and even UHT (120° to 140°C) temperatures.[35,43,46,47,141,194,221] Griffiths et al.[103] showed that both proteases and lipases produced by psychrotrophs, representative of a number of genera, retained 60% to 70% of their activity after heating at 77°C for 17 seconds and about 30% to 40% of their activity remained after UHT treatment at 140°C for 5 seconds. The heat stability of these enzymes is influenced by milk proteins, which exert a stabilizing effect.[134] Not all the psychrotrophs found in milk are capable of synthesizing these enzymes and, even in those that do, the levels produced are affected by many factors, including growth phase, nutrient supply, and environmental conditions, such as temperature, oxygen tension, etc.[72,125,160,194] In addition to proteases and lipases, certain psychrotrophic bacteria produce other extracellular enzymes that affect milk quality such as phospholipase C and glycosidases. The role of these enzymes in the degradation of milk fat was discussed in Section 22.2.2.

22.4.7 Public Health Significance of Raw Milk

Regulations determining the sale of raw milk differ from country to country but, even in jurisdictions requiring mandatory pasteurization of milk, pressure is being placed on the regulatory agencies to relax their restrictions on the availability of this product to the public. This has been driven by the consumers' desire for "natural" foods and the perceived nutritional advantages offered by raw milk. Despite the demands of a vociferous minority, the question remains: Can the safety of raw milk be guaranteed? The answer to that question must undoubtedly be no. There is a vast literature demonstrating the efficacy of pasteurization in reducing milkborne illness and an equally weighty volume of literature documenting illness produced through the consumption of raw milk.[195] In England and Wales during 1985 to 1989, the great majority of outbreaks of milkborne illness were associated with the consumption of raw milk, although it accounted for less than 1% of sales. In Scotland, a similar situation existed until the sale of unpasteurized milk was prohibited in 1983. After this time the incidence of disease associated with liquid milk dropped significantly and was further reduced when legislation was introduced in 1986 to prohibit farm workers from receiving untreated milk as part of their wages (Figure 22-2).[30] The higher number of cases observed in England and Wales may simply reflect differences in population levels. The decrease in cases in England and Wales in 1988 may have been due to an increase in vigilance within the industry following an outbreak of salmonellosis linked to infant formula in 1985, which involved 62 cases and one death. This had severe repercussions for the company that produced the formula and resulted in the Dairy Trade Federation formulating guidelines for the hygienic production of milk products.

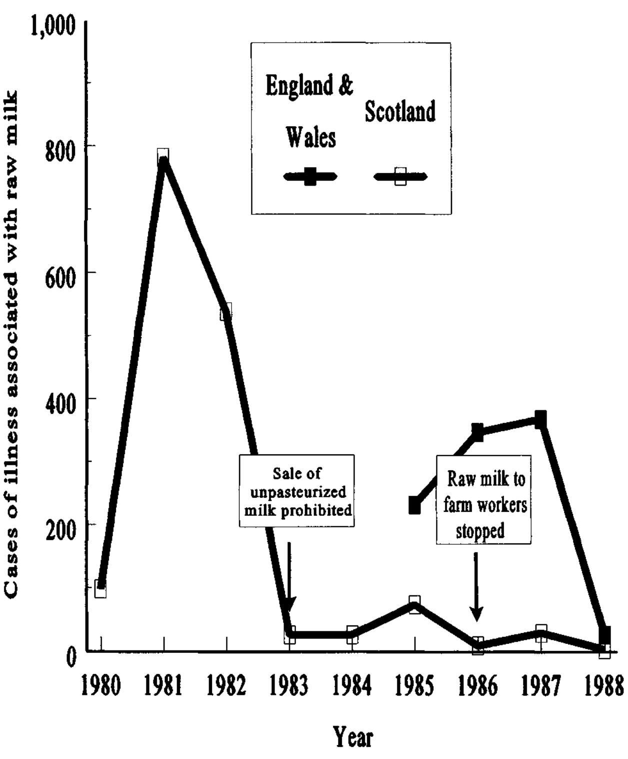

Figure 22-2 Effect of Regulatory Changes on the Incidence of Milkborne Illness in the UK. *Source:* Data from R. Burt and S. Wellsteed, Food Safety and Legislation in the Dairy Industry, *Journal of the Society of Dairy Technology*, Vol. 44, pp. 80–86, 1991.

Further evidence to indicate the hazard associated with consumption of raw milk was produced during a study of foodborne infections conducted in Ontario between 1979 and 1985.[245] Cases of salmonellosis, campylobacteriosis, and yersiniosis were more prevalent in rural areas, and it was concluded that raw milk played an important role in these infections.

Another index of the dynamic nature of milkborne illness is the changes observed in the etiological agents with time. In the middle of the 20th century, the main illnesses associated with the consumption of milk were brucellosis and tuberculosis. These have been eradicated as milkborne illnesses in developed countries, mainly through herd certification programs, the installation of refrigerated bulk tanks for collection of milk on farms, and the introduction of pasteurization. The majority of present-day milkborne illnesses are attributable to *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* among others and are associated with the consumption of raw milk or pasteurized milk that has either received an inadequate heat treatment or has been contaminated after heating. These epidemiological changes have been brought about by the adoption of new milk production, processing, and distribution practices. Other factors, for example, the changing characteristics of microorganisms and demographic changes such as the ageing population and increase in numbers of immunocompromised individuals, will ensure that this dynamism will continue.[71]

The important pathogens present in raw milk have recently been reviewed in a publication of the International Dairy Federation.[123] Several surveys have been conducted, worldwide, to determine the incidence of pathogens in raw milk. The results of some of the more recent surveys are summarized in Table 22-4. What is obvious is that traditional indices of the microbiological quality of milk, such as aerobic plate counts, cannot be relied upon as a guarantee of the safety of the milk. Pathogens can be isolated from low count milk produced under hygienic conditions.[56,232]

As well as the more common pathogens associated with raw milk, recent attention has focused on the potential of raw milk for the transmission of other organisms. For example, Farrell and Marth[80] have suggested that raw milk may be a vehicle for the transmission of *Borrelia burgdorferi*, the agent responsible for Lyme disease. It has been shown that the organism can survive for at least 46 days in milk stored at 5°C.[81] Also, *Mycobacterium paratubercu-*

Table 22-4 Incidence of Potential Bacterial Pathogens in Raw Milk from Some Surveys Published between 1990 and 1995

Organism	*Incidence in Ontario Bulk Tank Milk*[a] *% Samples +ve*	*Incidence % of Raw Milk +ve*[b] *(mean)*	*References*
Aeromonas hydrophila	—[c]	35.3	122
Campylobacter jejuni	0.46[d]	1.4–12.3 (6.7)	55, 139, 207
Clostridium perfringens	—	0–9.3 (4.7)	55,172
Listeria monocytogenes	2.84	0–15.6 (6.9)	55, 82, 110, 122, 170, 171, 183, 207
Salmonella spp.	0.17	0.1–8.9 (3.1)	55, 183, 203, 207
Staphylococcus aureus	—	62	55
Verotoxigenic *Escherichia coli*	0.81		
Yersinia enterocolitica	—	15.1–36.0 (24.5)	55, 107, 140, 207

[a] Unpublished data from Ref. 232.

[b] Incidences reported in the literature between 1990 and 1995.

[c] Isolation of the organism was not included in the study.

[d] *Campylobacter* spp.

losis, suspected to be the etiological agent of Crohn's disease, has been isolated from 11.6% of milks from asymptomatically infected cows.[242] Recently, verocytotoxigenic strains of *Escherichia coli*, particularly the serotype *E. coli* O157:H7, have caused severe food-related outbreaks. Although the primary source appears to be inadequately cooked beef burgers, dairy cattle have been shown to be a major reservoir for infection, and cases linked to the consumption of raw milk have been reported.[63] These have included cases among the families of dairy farmers. *E. coli* O157:H7 is unable to grow in milk at a temperature of 5°C and below and about a 2 log cycle decrease in count is observed after 28 days at this temperature.[271] However, significant growth (1 to 2 log cycles) can occur within 4 days when the milk is stored at 8°C. The organism grows more slowly in unpasteurized than pasteurized milk, presumably because of the antagonistic effect of the native flora and the antimicrobial activity of raw milk.

It is not only bacterial contamination of milk that causes concern. There has been a number of large outbreaks of cryptosporidiosis, an illness produced by the protozoan *Cryptosporidium parvum* (see Chapter 51). Whereas these have mainly been waterborne, there is substantial epidemiological evidence to implicate raw milk as a vehicle.[136] However, a survey of over 100 raw milk samples from Ontario failed to detect *Cryptosporidium* oocysts (Laberge and Griffiths, unpublished results).

22.5 NATURAL ANTIMICROBIAL COMPOUNDS IN RAW MILK

Raw milk is known to contain several antimicrobial compounds. The role of many of these is to confer a degree of protection on neonates of the species from which the milk was obtained and to protect the mammary gland itself from infection. However, these compounds can also play a role in the preservation of milk.

22.5.1 Lactoperoxidase

Arguably, the most extensively studied of the antimicrobial systems found in milk is lactoperoxidase. This enzyme catalyses the oxidation of thiocyanate, in the presence of hydrogen peroxide, to give an intermediary oxidation product, hypothiocyanite, which has antibacterial activities due to oxidation of sulfhydryl groups of proteins and structural damage to the cytoplasmic membrane of bacteria.[272] Thiocyanate is present in a wide variety of animal tissues and secretions, including milk, and the hydrogen peroxide can be generated endogenously by the addition of H_2O_2-forming bacteria such as lactobacilli and lactococci, by the oxidation of ascorbic acid, or by the oxidation of glucose by glucose oxidase.

The lactoperoxidase system (LP-S) inhibits many bacterial species, including milkborne spoilage and pathogenic microorganisms. Its bactericidal action against Gram-negative bacteria, including pseudomonads,[21] has been used to extend the storage life of refrigerated, bulk tank milk.[20] While it has not gained widespread use in developed countries, there are reports on its application in Third World countries. Lactoperoxidase also exerts an inhibitory effect on Gram-negative pathogens, such as *Aeromonas hydrophila*,[214] *Campylobacter jejuni*,[24] *E. coli*,[66,204] including *E. coli* O157:H7,[78] *Salmonella* spp.,[66,199,204] and *Yersinia enterocolitica*.[79] Despite the fact that its effects on Gram-positive bacteria are mainly bacteriostatic, LP-S is useful for the control of the growth of *Listeria monocytogenes*[19,54,64,70,86,130,227] and *Staphylococcus aureus*[130] in milk. The inhibition produced by the LP-S is affected by the metabolic status of the organism, the growth medium, and environmental factors such as pH and temperature. Several methods by which bacteria can be resistant to the effects of LP-S have also been described.[272]

22.5.2 Lactoferrin

Lactoferrin (Lf) is an iron-binding glycoprotein found in milk, which inhibits the growth of many bacteria by its ability to chelate iron.[142] As well as the inhibition caused by its ability to sequester iron, iron-free Lf (apo-Lf) can exert a bactericidal effect through direct interaction with bacterial cell surfaces.[7] The antibacterial properties of apo-Lf are unaffected by pasteurization but are lost upon UHT treatment of milk.[189] The antibacterial effect of Lf has been shown to increase when the glycoprotein is hydrolyzed by heat at acidic pH[211] or by pepsin digestion.[13,260] The inhibition of microbial growth produced by this hydrolyzed product, known as lactoferricin, is not mediated by iron chelation.[211] Lactoferricin is active against a wide variety of organisms, including members of the Enterobacteriaceae, pseudomonads, *Bacillus* spp., and *L. monocytogenes*.[14,128,269] The practical significance of lactoferricin is uncertain.

22.5.3 Lysozyme

Lysozyme is a bacteriolytic enzyme, present in raw milk, that hydrolyzes mucopolysaccharides in bacterial cell walls.[36] The enzyme is capable of surviving HTST pasteurization.[94] Lysozyme is mainly active against Gram-positive bacteria and has been used for the control of late-blowing of cheese by *Clostridium tyrobutyricum*.[33] However, in the presence of chelating agents such as EDTA, lysozyme can exert a significant inhibitory effect on some Gram-negative bacteria.[190,202] The addition of hen egg lysozyme to milk did not have a significant influence on the shelf life achieved,[101] and Stadhouders et al.[230] reported that lysozyme was only effective when spore populations in milk were low (about 3 spores 10 ml^{-1}). However, milk lysozyme may be more effective as an antimicrobial agent than the enzyme from egg.

22.5.4 Immunoglobulins

Bovine milk, especially colostrum, is relatively rich in immunoglobulins and use has been made of this richness through the concept of "passive immunotherapy."[191] Immunoglobulins isolated from bovine milk have been found to reduce incidence of infection by *E. coli*[244] and rotavirus.[67,116] Indeed, raw milk contains an antiviral compound, identified as IgG,[243] which is very effective against rotavirus. It remains to be seen whether there are significant differences between growth rates of pathogens in raw milks containing immunoglobulins directed at the organism of concern and growth rates in raw milks that are free of such antibodies.

22.6 COMPETITIVE EFFECTS BETWEEN MICROORGANISMS FOUND IN MILK

The inhibitory effects of lactic acid bacteria on growth of competing microflora are well documented, and the addition of these organisms to raw milk to increase shelf life prior to processing is an accepted dairy industry practice.[35] The inhibitory effect is mediated through a combination of reduced pH, production of organic acids, and the synthesis of antimicrobial compounds (bacteriocins) by the lactic acid bacteria. However, the most important factor for controlling the growth of psychrotrophs in milk by the addition of lactic acid bacteria seems to be acid production.[98] This technique can only be used for the extension of storage life of milks used for the manufacture of fermented dairy products; therefore, it will not be considered further.

There have been reports that the growth of *Listeria monocytogenes* is influenced by the presence of Gram-negative psychrotrophs such as *Pseudomonas* spp.,[154] *Flavobacterium* spp.,[76] and *Yersinia enterocolitica*.[29] The growth of *L. monocytogenes* was accelerated in media that had previously supported growth of pseudomonads; the effect was mediated through the production of proteases by the pseudomonad.[154] However, when *L. monocytogenes* was grown in the presence of *P. fluorescens* and other Gram-negative psychrotrophs, no such synergistic effects were observed,[74,75,77] presumably due to the fact that *L. monocytogenes* could reach significant levels before the production of proteases by the Gram-negative psychrotrophs. It was concluded that *L. monocytogenes* was an effective competitor with the common microflora of milk when grown at low temperatures. Farrag and Marth[76] determined that the growth of *L. monocytogenes* was enhanced in the presence of *Flavobacterium lutescens* but was inhibited by another species of *Flavobacterium*. *Yersinia enterocolitica* also inhibited *L. monocytogenes* during growth at 10° and 22°C in mixed culture, but the inhibitory effect was not apparent at 4°C.[29]

Mesophilic strains of *Bacillus* spp. (mainly *B. subtilis* and *B. licheniformis*) produce factors that are antagonistic to the growth of psychrotrophic strains of the same genus.[241] The significance of these interactions is not entirely clear but they may influence the incidence and growth of psychrotrophic strains in the farm environment.

22.7 OTHER METHODS FOR PROLONGING THE STORAGE LIFE OF RAW MILK

22.7.1 The Need To Prolong the Storage Life of Milk

As stated earlier in this chapter, because of changes in management practices at the farm and at the dairies, raw milks are being subjected to longer storage times before processing. This has led to the development of methods to extend the storage life of raw milks. The two methods that have gained greatest acceptance both involve the use of temperature: either heating or cooling.

22.7.2 Thermization

Thermization is a heat treatment, usually in the range 60° to 65°C for 10 seconds, designed to reduce the psychrotroph count to levels that will allow storage of the milk at 4°C for 3 to 5 days. The process can be carried out on the farm or at the processing site. In a study of the effects of heat treatment at sub-pasteurization temperatures on raw milk, Griffiths et al.[104] found that the temperature of thermization had a greater influence on storage life of raw milk at 4°C than the holding time. The ability to extend the storage life beyond 3 days was also determined by the initial psychrotroph count of the milk. If this count exceeded 1 × 10^4 cfu ml^{-1}, then to achieve a 3-day storage life required a combination of thermization and storage at 2°C.

22.7.3 Deep Cooling

Many workers have studied low temperature storage as a means of controlling the growth of psychrotrophs in raw milk.[35] By decreasing the storage temperature for raw milk from 6° to 2°C, a 2-day increase in storage life was observed for milk that had an initial psychrotroph count less than 1 × 10^4 cfu ml^{-1}.[105] The microflora of the milk "deep cooled" to 2°C was different from that stored at 6°C, with a greater preponderance of pseudomonads and lower proportion of Enterobacteriaceae in the milks stored at the lower temperature. Mathematical models have been produced that describe the growth of the microflora of milk as a function of temperature.[96]

22.7.4 Addition of CO_2

The growth of bacteria in milk can be inhibited by the addition of CO_2 at concentrations between 20 and 30 mM.[206] The addition of the gas caused a pH drop in the milk from between 6.59 and 6.7 to 6.2 and, following storage of the milks for 6 days at 7°C, there were significant differences in bacteria present in the treated and untreated milk (Table 22-5). The anaerobic count did not increase in the CO_2-treated milk. Similar extensions of storage life were obtained by King and Mabbitt[132] in an earlier study. There are concerns that CO_2 addition to milk can increase the heat resistance of spores present in milk.[106] As well as the effects on microbial growth, CO_2 may influence enzyme production by psychrotrophs. Rowe[209] found that lipase synthesis was inhibited more than the growth of *P. fluorescens* after

Table 22-5 Effect of CO_2 Addition at Concentrations between 20 to 30 mM on the Microflora of Milk Stored at 7°C for 6 Days

Population	*With CO_2 (cfu ml^{-1})*	*Without CO_2 (cfu ml^{-1})*
Aerobes	2.4 × 10^5	3.6 × 10^8
Coliforms	2.0 × 10^2	7.0 × 10^3
Facultative and obligate anaerobes	2.3 × 10^3	4.6 × 10^5

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the addition of 30 mM CO_2 to milk. The interest in the preservative effects of CO_2 in milk is continuing with its proposed use to extend the shelf life of pasteurized milk.

22.8 PROCESSING OF RAW MILK

22.8.1 The Need for Pasteurization

In many countries pasteurization (or equivalent heat treatment) is required before milk can be sold. The range of milk products available has also increased to meet consumers demands for products lower in fat. An increasing proportion of the fluid milk supply is homogenized as more milk is sold in cartons or pouches. Each of these steps has consequences for the microflora of milk. The effect of processing on the microbiology of milk will be discussed in the following sections.

22.8.2 Pasteurized Milk and Cream

Before milk is pasteurized it often undergoes cream separation. This is achieved by passing milk, warmed to 40° to 50°C, through a centrifugal separator. The milk temperature may vary depending on the equipment and plant policy. The procedure may remove some bacterial cells, especially those present in large clumps, but there is no evidence that the distribution of bacteria differs between the cream layer and the aqueous phase.[173]

Whole milk and cream are usually homogenized to disperse fat globules and, in the case of half and single creams, to increase viscosity. The homogenization step may help to break down clumps of cells. It also involves warming of the milk, so it is recommended that this step be carried out prior to any heat treatment of the product. Homogenization also makes the fat more susceptible to the action of lipases present in the milk. Lanciotti et al.[138] demonstrated that continuous homogenization at pressures between 400 and 2,000 bar resulted in an initial decrease in viable numbers of *Y. enterocolitica* and *L. monocytogenes* in milk, but the damage to the cells was reversible. However, the viability of vegetative cells of *B. subtilis* continued to decrease with time of storage of the milk at 3° to 4°C after homogenization, probably because the organism was not well adapted to growth at low temperatures. The natural microflora of the milk did not decrease upon subsequent refrigerated storage of the homogenized milk.

Flavor defects in milk and other dairy products can arise from four sources: (1) microbial growth in the milk prior to pasteurization, (2) activity of thermoresistant enzymes, (3) growth of thermoresistant organisms following pasteurization, and (4) post-pasteurization contamination.

Microbial Growth Prior to Pasteurization

Some of the factors affecting bacterial growth in raw milk have been addressed in previous sections of this chapter. It is generally recognized that bacterial counts of more than 1×10^6 cfu ml^{-1} are required before microbiologically related flavor defects are detected in milk.[35,194] However, the exact number of psychrotrophs necessary to produce off-flavors varies between species and is affected by growth rate and by the ability of the organism to produce extracellular enzymes that can degrade milk components.[46] Generally, in countries adopting good husbandry practices, the quality of the raw milk will rarely impinge on the quality of the final product. Ravanis and Lewis[201] determined that pasteurized milk with microbiological shelf lives of between 29 and 36 days at 4°C could be produced from raw milk that had been held for 9 days also at 4°C. At that point, the psychrotroph count in the raw milk was about 1.4×10^7 cfu ml^{-1}. Maximum shelf lives were obtained when the raw milk had been stored for 3 to 4 days but not when the milk was pasteurized after only 1 day of storage. They attributed this to the LP-S being maximally active under the former storage conditions. However, a major drawback to this study was that no organoleptic assessment of the milk was performed.

Activity of Thermoresistant Enzymes

The importance of the thermoresistance of the extracellular enzymes produced by many psychrotrophs has been addressed in several reviews.[35,43,46,72,141,160,194,231] The reader is referred to these for detailed information.

Growth of Thermoresistant Organisms in Milk Following Pasteurization

Pasteurization will destroy the majority of organisms associated with raw milk. Typical D- and z-values for some organisms commonly isolated from milk are shown in Table 22-6. There may be variations in the survival depending on the type of milk being pasteurized. Fat exerts a protective effect on Gram-negative bacteria and *L. monocytogenes.* The effect is more pronounced in sheep's milk than either cows' or goats' milk[151] but the protection was not sufficient to allow survival after current HTST pasteurization protocols. Nevertheless, there are

Table 22-6 Heat Resistance of Microorganisms Associated with Milk

Organism	D-value at 71°C in Milk (seconds)	z-Value (°C)
Pseudomonas fluorescens	0.001	4.4
P. fragi	0.26–0.32	6.9–7.6
Salmonella typhimurium	0.22	5.3
Campylobacter jejuni	0.02[a]	5.1
Yersinia enterocolitica	0.35–1.17[a]	5.1
Listeria monocytogenes	0.9	6.3

[a] Extrapolated value.

Source: Adapted with permission from J.D. Phillips and M.W. Griffiths, Pasteurized Dairy Products: The Constraints Imposed by Environmental Contamination, in *Food Contamination from Environmental Sources*, J.O. Nriagu and M.S. Simmons, eds., pp. 387–456, © 1990, John Wiley & Sons, Inc.

some bacteria that are capable of surviving pasteurization and growing in the heat-treated milk. Arguably, the most important of these are members of the genus *Bacillus*. The most frequently isolated species of *Bacillus* from pasteurized milk are *B. cereus* and *B. cereus*-group, *B. licheniformis*, *B. circulans*, *B. firmus*, and *B. subtilis*.[51,102,127,163,246] Of these, *B. cereus* and related species can grow at temperatures as low as 6°C[62,102] while other species, most notably *B. circulans*, can grow at 2°C.[102]

Psychrotrophic strains of *Bacillus* spp. have been isolated from about 70% of samples of pasteurized milk[102,163] and are capable of producing off-flavors in milk.[164] Of greater concern, however, may be the large percentage of toxigenic, psychrotrophic strains of *B. cereus* that can be isolated from milk and that have been shown to be capable of producing enterotoxin during growth in refrigerated milk.[41,95,266] Growth of these bacteria in creams was always associated with obvious spoilage but, in flavored desserts, spoilage was not always evident before high numbers of *B. cereus*, with consequent toxin production, were reached.[240] Other *Bacillus* spp., including *B. licheniformis*, *B. subtilis*, and *B. pumilus*, all of which have been isolated from pasteurized milk, have been implicated in foodborne illness.[97]

There has been much debate as to the source of the *Bacillus* spp. isolated from pasteurized milk. Crielly et al.[51] suggested that post-pasteurization contamination may not be the most important source of the organism, but their study did not involve typing of isolates. Evidence is accumulating, based on fatty acid profiling, biotyping, and genotyping, that *B. cereus* strains isolated from pasteurized milks are specific to the plant where the milk was processed[217] and that raw milk is the main source of these spore formers.[148] A study on the presence of *B. cereus* in a dairy plant showed that the number of spores in the milk decreased at each stage from the raw milk in the tanker, through storage, pasteurization, and packaging.[5] This decrease was explained by *B. cereus* spores adhering to surfaces throughout the processing chain. Thus, niches may occur in dairy plants downstream of the pasteurizer where *B. cereus* would proliferate almost unchecked and contribute to post-pasteurization contamination of the product.

The importance of thermoresistant psychrotrophs will increase as the move toward extended shelf life products gains momentum. In these heat-treated, aseptically packaged products, shelf life is mainly limited by *Bacillus* spp.[22,52] Cromie[52] studied milk processed at between 72° and 88°C and concluded that, when milk was stored at 3°C, the milk heat treated at 72°C had the longest shelf life but the difference was not as great when milk was stored at 7°C. The faster growth in milk processed at the higher temperatures could be due to spore activation, destruction of natural milk antimicrobial systems, or reduced competition from other bacteria. When extended shelf life milk was produced by heat treatments at between 100° and 145°C, *Bacillus* spp. were isolated from milk processed at and below 132°C but no organisms were isolated from milk heated at 134°C and above.[22]

Although no definite link has been verified, there is evidence that *Mycobacterium paratuberculosis* is linked to Crohn's disease. There is now substantial evidence to suggest that this organism can survive pasteurization.[38,92,241] Recent work has cast doubt on these findings as, although *M. paratuberculosis* was able to survive temperatures between 65° and 76°C for up to 30 minutes when the heat treatment was performed in test tubes, the bacterium was effectively killed in raw milk heated at 72°C for 15 seconds in a laboratory-scale pasteurizer.[229] Further studies of the heat resistance of *M. paratuberculosis* in milk indicate that the levels of this bacterium that might occur in raw milk will not survive pasteurization.[131]

Post-Pasteurization Contamination

Although the Gram-negative, psychrotrophic bacteria present in raw milk do not survive pasteurization, the majority of studies on the microbiological quality of pasteurized milk and cream have reported the appearance of these organisms,[35,50,194] with *Pseudomonas* spp. being the most commonly isolated. Thus, it would appear that the shelf life of pasteurized products is limited by post-pasteurization contamination.

Milks with short shelf lives (<5 days at 4° to 6°C) contain almost entirely *Pseudomonas* spp. (90.7% of isolates), while milks with shelf lives >10 days have a higher proportion of other types of microorganisms (*Pseudomonas* spp. accounting for 68% of isolates).[100]

A variety of sources of contamination exist in the processing plant.[194] Personnel and air probably contribute little to the contamination of pasteurized fluid milk products. The normal microflora of air is dominated by Gram-positive bacteria, yeasts, and molds that do not grow well in milk at refrigeration temperatures. Vatne and Castberg[267] report the manufacture of good quality milk in dairies where the air contained aerobic counts as high as 2.8×10^3 cfu m^{-3}, although they recommend that the count should not exceed 1.5×10^2 cfu m^{-3} and the yeast and mold count should be less than 50 cfu m^{-3}. However, Ren and Frank,[205] following a study of the air quality of four fluid milk processing plants, concluded that personnel were a major source of biological aerosol in the dairy plant. This highlights the need for strict control, especially in packaging areas where there may be a safety concern.

With the concern raised by *L. monocytogenes* in dairy products, numerous surveys have been conducted into environmental sources for this organism in fluid milk plants.[224] In general, wet areas of the plants, such as conveyers, floors, drains, and case washers, were significantly more contaminated than dry areas. Although environmental samples were more frequently *Listeria*-positive than equipment swabs, environmental contamination (eg, floors, drains, air) did not necessarily mean that equipment would be contaminated within the same plant.[198] The plants were more likely to be contaminated if they were in close proximity to dairy farms.[197]

Contaminants are able to enter the pasteurized milk at any stage after the heat treatment. This contamination can even occur in the regeneration, cooling sections, and related pipelines of the pasteurizer itself.[4,222] This is especially true during extended operation of the pasteurizers beyond 8 to 9 hours.[144] After this period of time, bacterial numbers in pasteurized milk often rose exponentially and could reach $>1 \times 10^6$ cfu ml^{-1}. The increase was not seen in samples from the holding tube but occurred in milk sampled from the cooling side of the regeneration section. The rate of bacterial contamination was influenced by the raw milk quality which, in turn, was related to temperature and flow characteristics of the raw milk in prepasteurization balance tanks. These bacteria were mainly thermoduric streptococci that did not necessarily influence the shelf life of the product because of their inability to grow at low temperatures.[25] Potentially, the most dangerous form of contamination contributed by the heat exchanger is adulteration of the pasteurized product by raw milk through leakage in the regeneration section because of corrosion.[16] Improper operation, allowing inadequate heat treatments, is also a serious cause of contamination of the final product, which has resulted in outbreaks of milkborne illness.[194]

Contamination of the heat-treated milk can occur downstream of the heat exchanger. This contamination can arise from milk pipelines and holding tanks. The role of airborne contaminants in the contamination of pasteurized milk has been the subject of much debate[194] but may be an important contributor especially during the emptying of storage tanks. A more likely cause of post-pasteurization contamination is shedding of bacteria from biofilms formed on gaskets in pasteurized milk pipelines. Electron microscopy has been used to show that biofilms can develop on the sides of gaskets, despite operation of clean-in-place systems.[8] It is well accepted that sanitizer efficacy is greatly reduced against bacteria growing in biofilms.[169] However, other workers, using radiolabeled bacteria, concluded that milk inhibited adherence of the bacterial strains studied to steel, rubber, and glass. They suggested that intrinsic bacterial adherence did not contribute significantly to the contamination of dairy equipment.[237]

Other factors may contribute to survival of organisms on gaskets. For example, Pfeifer and Kessler[192] provided evidence that the heat resistance of *B. cereus* spores increases when they are present in an enclosed space between a silicone rubber seal and a stainless steel surface. This would be the case with gaskets. They postulate that the increase in heat resistance is due to the reduced water activity of the environment.

There is substantial evidence that the filling operation has the greatest influence on the potential shelf life of pasteurized milk.[99,168,218] Low levels of bacteria (10 to 500 psychrotrophs $liter^{-1}$) can be introduced into the product at this stage, which can have a profound effect on shelf life.[100] Continuing improvements to the design of fillers have resulted in dramatic effects on the keeping quality of milk.[219] The influence of aseptic packaging of milk on the expected shelf life was amply demonstrated by Schmidt et al.[216] who demonstrated that this milk could be kept for about 48 days when stored at 3°C. Pasteurized milk is not usually aseptically packaged, whereas the procedure is necessary for UHT milks to maintain "commercial sterility."

The packaging material can also have an influence on the shelf life of pasteurized milk either by direct contamination of the product by organisms on the material, or indirectly by allowing bacteria to penetrate into the product.[150]

22.8.3 UHT Products

Milk that has undergone UHT treatment is "commercially sterile," although certain spore formers, especially species of thermophilic *Bacillus*, are able to survive the process. However, they generally do not limit the shelf life of the product because they are unable to grow much below about 40°C. It is accepted that the major cause of failure in UHT products is due to packaging problems associated with the aseptic filling techniques. A comprehensive review of UHT processing has been published.[31]

Recent problems have been encountered in Europe because of the ability of certain highly heat-resistant spores of mesophilic *Bacillus* spp. to survive the UHT process and grow during testing under the new European Union (EU) Regulation 92/46.[108,145] Although they do not compromise shelf life or safety, they cause "false" failures under the regulations.

Prior growth of Gram-negative psychrotrophs in raw milk can have a significant effect on the sensory acceptance of UHT milk because of the ability to produce thermostable extracellular enzymes. The psychrotrophic count in raw milk was strongly correlated ($r = 0.95$) with the extent of proteolysis in stored UHT milks.[45] Correlations between the level of proteolysis and bitter flavors in stored UHT milk were high when the milk was stored at 30°C ($r = 0.90$) and at 40°C ($r = 0.92$), but not when the UHT milk was stored at 20°C ($r = -0.23$). Length and temperature of storage had a greater effect on the sensory quality, and hence shelf life, of the UHT milk than the psychrotrophic count of the raw milk.

22.8.4 Condensed/Evaporated Milk

Sweetened Condensed Milk

With the technology currently employed to produce sweetened condensed milk, microbiological defects are relatively uncommon. The problems that do arise are almost invariably due to contamination of the milk following forewarming. The most common microbiological problems are gas formation from the growth of the yeast, *Torulopsis*, or, more rarely, growth of coliforms. The defect usually appears slowly because of the slow growth rate of the organisms at the high sugar concentration of the product and results in blowing of the container.[180] Many species of bacteria, particularly micrococci and *Bacillus* spp., can cause thickening in condensed milk of low sugar content. Mold growth and coagulated casein can also be observed on the surface of opened cans that have been stored for long periods of time. Both *Penicillium* and *Aspergillus* spp. have been implicated. The thickening and mold growth are generally the result of contamination from the plant environment and can be controlled by storing the product at lower temperatures, as well as improving plant sanitation.[180]

Evaporated Milk

Evaporated milks are produced by an aseptic canning process and, hence, are commercially sterile. Problems are encountered only when heat treatments are inadequate or when organisms are introduced into the product through leaks in the hermetic seal of the can.[180] The most common defects are caused by *Bacillus* spp., and *B. stearothermophilus*, *B. coagulans*, *B. licheniformis*, *B. subtilis*, and *B. macerans* have all been isolated from evaporated milk with flat sour spoilage.[129] When cans are opened by the consumer, contamination is invariably introduced and the product becomes perishable.

22.8.5 Dried Milks

The emphasis on the production of dried milk powders has turned toward the manufacture of products with specific properties and to minimizing treatments in order to optimize the nutritional value of the end product. These trends have increased the need for reliable microbiological control. The low water activity of milk powders precludes the growth of microorganisms and, generally, bacterial numbers in powdered milk decrease during storage.[149] However, the microbiological quality of the raw milk used to manufacture dried milk can have a bearing on its properties, especially solubility, but only when the count in the raw milk is greater than 2×10^6 cfu ml^{-1}.[173]

As much of the milk powder manufactured is used for infant formula, particular attention must be focused on safety. Mettler[166] reviewed the requirements for pathogen control during the production of spray-dried milk powder and identified the following five critical rules: (1) prevention of pathogen entry into the plant, (2) prevention of growth in the event of pathogen entry, (3) pasteurization to reduce pathogens to a safe level, (4) prevention of post-pasteurization contamination, and (5) knowledge of the pathogen status of staff, premises, materials, equipment, and product. Far from being only applicable to the manufacture of powdered milk, attention to these points is crucial for the production of all nonfermented dairy products.

Despite the good record of the industry, outbreaks of foodborne illness associated with the consumption of milk powders have been documented.[166] Of the 24 outbreaks cited by Mettler,[166] 20 were due to *Salmonella* spp., 3 were caused by staphylococcal enterotoxin, and 1 was associated with *Clostridium perfringens*. Salmonellae can survive spray drying, and there is evidence that contamination by both *Salmonella* spp. and *Staphylococcus aureus* may take place during manufacture.[149] In response to this, the microbiological standards for milk powders are continually being tightened.

Concern has been raised about the presence of *B. cereus* in dried milk products, especially infant foods.[11,51] Spores can survive in powders for at least 6 months[173] and both Holmes et al.[118] and Kramer and Gilbert[133] have cited outbreaks of *Bacillus*-related foodborne illness associated with milk powder and infant formula. This concern may be heightened by results indicating that about 50% of samples of infant formulae were contaminated with *B. cereus*[11] and that these organisms could grow rapidly in reconstituted milks stored at ambient temperatures.[11,51] The temperature of reconstitution also played a role in the rate of growth during storage, with growth being most rapid in powders reconstituted at 45°C.[11]

22.8.6 Butter

Although butter contains not less than 80% fat, not all of the water is present as discrete droplets expected in an ideal water-in-oil emulsion; some exist as minute continuous channels in which microorganisms can proliferate.[10] In such a compartmentalized food, microbial growth is proportional to the availability of nutrients and the radius of the water droplets.[268] The main cause of spoilage of butter is lipolytic rancidity. In cold-stored, sweet-cream butter, only organisms that can survive low temperatures and high salt concentrations are found with micrococci generally predominating. However, *Pseudomonas* spp. may be introduced from wash water and cause a surface taint.[178]

There have been outbreaks of illness caused by staphylococcal enterotoxin in butter[17] and *Staphylococcus intermedius* was identified as the causative agent in one outbreak where butter was the vehicle of transmission. *Listeria monocytogenes* survived and grew in butter made in a small, home-style churn.[184] This may not be indicative of the organism's ability to survive the commercial buttermaking process where the moisture droplets in the butter may be smaller.

The trend toward low-fat (or light) butter is disturbing from a microbiological standpoint. Lanciotti et al.[137] observed that the yeast, *Yarrowia lipolytica*, and the bacteria, *Enterococcus faecium* and *Bacillus polymyxa*, were the organisms most commonly isolated from commercial packages of light butter. They also isolated *Bacillus firmus*, *B. laterosporus*, and a yeast, *Cryptococcus albidus*. Of greater concern is the ability of *L. monocytogenes* and *Yersinia enterocolitica* to grow in this product. *Listeria monocytogenes* displayed a higher growth potential than *Yersinia*, possibly due to it being less affected by space and nutrient limitations in this compartmentalized food.[137]

22.8.7 Ice Cream

The microbiology of ice cream has been reviewed by Rothwell.[208] He divided the factors that affect the microbiological quality of ice cream into three components: (1) the quality of the raw materials, (2) the efficiency of processing, and (3) hygienic conditions under which the ice cream is produced. Of the various materials that can be used to produce ice cream, many can contribute to the quality and safety of the final product. However, proper handling and storage will eliminate the majority of problems.

Ice cream mix is a highly viscous product. Concern has been expressed that laminar flow conditions are set up during HTST pasteurization, resulting in underheating of the product, even at the higher pasteurization temperatures usually used to process this product.[89] That laminar flow is achieved during HTST pasteurization of ice cream mix was confirmed by Davidson et al.[53] In addition, it has been found that the heat resistance of *Listeria monocytogenes* is increased by common stabilizers used in ice cream mix, such as guar gum and carrageenin.[119] Thus, particular attention should be paid to ensure that the mix receives an adequate heat treatment.

As well as the use of high quality raw materials and provision of an adequate pasteurization process, proper attention must be paid to the cleanliness and sanitation of the processing plant, plant personnel, and dispensing equipment. The latter is a particular cause for concern for soft-serve ice cream, which is sold from a special dispensing freezer. These dispensers must be properly cleaned and disinfected at frequent intervals and problems due to the presence of *L. monocytogenes* in these products are still evident. Post-pasteurization contamination of ice cream products by *Listeria* can occur via aerosol formation even at subzero temperatures.[90]

Many pathogens, including *Listeria monocytogenes* and *Yersinia enterocolitica*, can survive freezing.[69,261] In recent years, ice cream has acted as a vehicle for the transmission of listeriosis, salmonellosis, and *Staphylococcal* enterotoxin poisoning.[208] A recent large outbreak of salmonellosis was attributed to ice cream that had become contaminated during transportation of ice cream mix in a truck previously used to carry raw liquid egg. This incident highlights the need for vigilance during all aspects of production of not only ice cream, but all food products. *Bacillus cereus* has also been isolated from a large number of ice cream samples (48% in one survey[1]) and outbreaks attributed to the presence of *B. cereus* in ice cream have been reported.

22.9 OTHER METHODS OF PASTEURIZATION OF MILK

22.9.1 The Need for Alternatives to Heat Treatment

Because of possible nutritional losses and organoleptic changes associated with heat pasteurization, along with high energy costs, there has been much interest in the development of nonthermal pasteurization methods. A number of these are under investigation, though none have replaced HTST treatments to date.

22.9.2 Microfiltration

Using ceramic membranes with pore sizes of 1.4 µm, a system has been developed that allows the sanitary cross-flow filtration of milk. This method, termed microfiltration, has been shown to reduce bacterial numbers in nonfat milk by up to 3 log cycles[68,263] and *Listeria innocua* in milk by about 2 log cycles.[152] Milk treated by combination of a microfiltration process with a minimum HTST pasteurization (about 72°C for 16 seconds) is currently sold in Canada. The thermal pasteurization step is still a statutory requirement and cannot be omitted. Before microfiltration the milk is skimmed and the fat is heat treated separately before recombination with the microfiltered, HTST pasteurized skim to produce a milk with the desired fat concentration. The products have an extended shelf life of at least 32 days at 4°C, and an improvement in organoleptic quality is claimed.

22.9.3 Bactofugation

Microorganisms can be removed from milk by centrifugation in a process called bactofugation. The method is claimed to be effective at removing bacterial spores[226] but it can be plagued by problems with recontamination. An extended shelf-life milk produced by combining bactofugation and HTST pasteurization is on sale in Canada. It has an expected shelf life of at least 30 days at 4°C.

22.9.4 Irradiation and Microwave Treatment

Sadoun et al.[210] proposed that a low-dose irradiation of milk was possible without affecting organoleptic or nutritional properties. A treatment of 0.25 kGy at room temperature doubled the shelf life of pasteurized milk. Irradiation at 0.5 kGy produced a 2 log cycle reduction in bacterial count of raw milk, but the changes in the sensory properties of the milk were such that the treatment had little effect on storage life.

Microwave heating has also been shown to have limited application for milk.[112]

22.9.5 Ultrasound

The antimicrobial effects of ultrasound (or sonication) have been known for over a century but there has been limited work carried out on its application in the food industry. Ultrasound mainly works by cavitation, the production of bubbles or cavities in a liquid.[215] Cavitation can be either stable, where the bubbles remain intact and damage cells by shear forces, or it can be transient, causing large pressures to be produced when the bubbles collapse.[65] These large pressures disrupt cell-wall structures, resulting in irreparable damage to the cell. Early work conducted on the pasteurization of milk by ultrasound produced disappointing results but bacterial cells become more sensitive to subsequent heat treatments after sonication.[65,212] This effect may be utilized to reduce the temperatures required to adequately pasteurize milk products.

22.9.6 High Hydrostatic Pressure

The availability of commercial equipment for high-pressure processing of foods has revived interest in this technique as an alternative to thermal pasteurization.[165] Pressure causes protein denaturation and disrupts the structural integrity of cells.[65,179] However, the problem posed by recovery of cells sublethally damaged during hydrostatic pressure treatments must be overcome. The answer may lie in the development of combined treatments as proposed for sonication. Spores may also survive high pressure but the treatment seems to activate them and pressure-germinated cells could be subsequently eliminated by an alternative treatment.[65]

22.9.7 High Voltage Electric Pulses

Use of high voltage electric pulses (HVEP) is one of the most promising techniques currently under investigation for the nonthermal pasteurization of milk.[200,223] Qin et al.[200] cite research that used HVEP to successfully pasteurize raw milk with a peak electric field of 40 kV cm^{-1} and 20 pulses each of 2 μsec duration. This represents a significant reduction in cost over thermal pasteurization techniques.[117] Reversed polarity of the pulse appears to be necessary for optimal antimicrobial effects[117] and there are preliminary reports that this technology is also lethal to spores.[157]

22.10 THE FUTURE

Many large outbreaks of disease transmitted by milk or milk products have occurred in recent years, but these have invariably been due to the consumption of raw milk, milk that had received an inadequate heat treatment, or milk that had been contaminated after pasteurization. Nevertheless, both producers and processors have made significant strides to improve the quality and safety of dairy products but the amazing adaptability of microbial populations means that the challenge to maintain standards will not lessen. Indeed, as new technologies are adopted, different problems will ultimately emerge. To deal with potential threats to the microbiological safety and quality of milk products, the production, processing, and retailing components of the industry must work together to develop integrated quality management and hazard analysis critical control point (HACCP) schemes. It is only by concerted action that the high standards set by the dairy industry in the past can be maintained.

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23

Fermented Milk Products

Michael Teuber

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> *. . . as fig-juice thickens quickly the white*
> *milk, the fluid, that curdles quite promptly*
> *through stirring . . .*
> *Homer's Iliad V.902–903*

23.1 HISTORICAL INTRODUCTION

Agriculture and pastoralism formed the basis for the neolithic revolution about 7,000 years BC, ie, for the transition of a mostly nomadic way of living of hunters and gatherers into a sedentary life. The domestication of farm animals was initially a method of keeping live meat (protein) reserves. Evidence from excavated bone remains from settlements of about 5,000 BC indicate an increasing number of adult female animals. This provides the first circumstantial evidence that sheep, goats, and cattle were also kept for milking. We have no physical evidence for fermented milk products from prehistoric times.[54] The invention of writing about 3,200 BC by the Sumerians started with bookkeeping documents of agricultural products and commodities. These documents already contained signs for dairy products such as milk, butter, fat, cheese, and yogurt obtained from goats, sheep, and cows.[128] The assumptions on how these early dairy products were made by spontaneous fermentation follows.[40,135]

Acknowledgments. I would like to thank Ulrike Wollert for the tedious typing work, and specifically Dr. Barbara Lund for her expert editing of my manuscript and her patience.

full fat milk
↓ *spontaneous fermentation*
yogurt
↓ *addition of water + churning*
butter + **butter milk**
↓ *heating* ↓ *cheese cloth* → **whey**
butter oil **curd**
↓ *drying*
dried cheese

Butter oil and dried cheeses can be kept for years because they are microbiologically stable as long as they are kept in dry conditions (Table 23-1).

Later literary documents from Greece (eg, Homer's *Iliad* and *Odyssey*) and Palestine (*Old Testament* of the *Bible*) contain evidence of the existence of sour milk and cheeses. The fermentation of milk into such products seems to be an ancient heritage of human culture. This was only possible because the milk provided quite specific conditions for lactic acid bacteria to cause fermentation sufficiently rapidly to limit the growth of spoilage bacteria (eg, bacilli, pseudomonads, or clostridia). However, it was only in 1856 that Louis Pasteur realized that the lactic fermentation of milk was a microbial process.[104] It then took only a few decades to isolate and describe the main bacterial species responsible for lactic fermentations, ie, the mesophilic and thermophilic lactic acid bacteria with coccoid and rod-shaped morphologies. The first of these bacteria obtained in pure culture was *Bacterium lactis*, now called *Lactococcus lactis*.[84] The first bacterial starter cultures for dairy fermentation isolated from spontaneously fermented milk and cheese were available around 1900 in Denmark, Germany, and the United States. These starter cultures were provided as liquid cultures in milk. After World War II, freeze drying was employed to prepare cultures that were stable in vacuum-sealed containers for many months and could be shipped at ambient temperatures to all parts of the world. Deep-frozen, concentrated cultures which, like freeze-dried concentrated cultures, can be applied for direct inoculation of fermentation tanks developed about 20 years ago. The microbiological development of dairy starter cultures culminated in the application of the principles of genetic engineering to lactic acid bacteria within the last 10 years.[48] Genetically modified lactic acid bacteria (eg, with modified phage resistance, bacteriocin production, proteolysis, lactose fermentation) are ready to be applied in practice as soon as the legal, environmental, and health issues are resolved. The most recent development is the application of lactic acid bacteria as "probiotics" and antigen-delivery systems for vaccination purposes.[144]

Table 23-1 Estimated Keeping Times of Dairy Products at Ambient Temperatures (20° to 30°C)[135]

Keeping Time	*Milk and Its Protein Fraction*	*Fat Fraction*
½ day	raw milk	sweet cream
1 day	boiled milk	—
1 week	sour milk	sour cream
2 weeks	sour curd/fresh cheese	butter
2–3 weeks	soft cheese	—
1–2 years	hard cheese	—
Several years	dried cheese	butter oil

Source: Reprinted with permission from M. Teuber, How Can Modern Food Technology Help To Identify Dairy Products Mentioned in Ancient Sumerian Texts? *Bulletin of Sumerian Agriculture*, Vol. 8, pp. 23–31, © 1995, University of Cambridge.

The scope of this chapter is to summarize the microbiological principles of modern dairy fermentations with brief references to the underlying biochemical, genetic, ecological, technological, and hygienic aspects.

23.2 THE SUBSTRATE MILK[136]

Milk of several animal species is used for fermentations into sour milk and cheese (Table 23-2). The main biochemical components necessary for the lactic acid bacteria are lactose as a carbon and energy source, the casein fraction as a nitrogen source, and citrate as a substrate for diacetyl production. The free amino acid fraction of cow's milk, for example, is too low to provide for cell densities higher than 10^7 cfu ml^{-1} for nonproteolytic bacteria.

Specific indigenous antimicrobials in milk, including the lactoperoxidase/thiocyanate/hydrogen peroxide systems, lysozyme, and iron-binding lactoferrin, probably inhibit Gram-negative, facultative bacteria more than the Gram-positive anaerobic or microaerophilic lactic acid bacteria. However, these systems are only active in raw milk because they are denatured by pasteurization.

During milking, contamination of raw milk with microbes derives from the environment (skin, udder, feces, air, fodder, equipment) and excretion with milk if the animal suffers from latent or acute mastitis or other infective diseases.[131] In the dairy there is a risk of further pre- and post-pasteurization contamination. Therefore, the production of microbio-

Table 23-2 Composition of Milk from Different Animal Species (Average Values, Undefined Lactation Phases) (% w/v)

Animal Species	*Dry Matter %*	*Water %*	*Fat %*	*Casein %*	*Whey Protein %*	*Lactose %*	*Ash %*
Cattle	12.7	87.3	3.7	2.8	0.6	4.8	0.7
Goat	13.2	86.8	4.5	2.5	0.4	4.1	0.8
Sheep	19.3	80.7	7.4	4.6	0.9	4.8	1.0
Buffalo	17.2	82.3	7.4	3.2	0.6	4.8	0.8
Camel	15.0	85.0	5.4	2.9	1.0	5.1	0.7
Horse	11.2	88.2	1.9	1.3	1.2	6.2	0.5

Courtesy of F. Puhan, Eidgenossische Technische Hochschule, Zurich, Switzerland.

logically safe milk for fermented dairy products requires careful control of every single production step. Pasteurized milk or milk reconstituted from milk powder (produced from pasteurized milk by spray or drum drying) are now the preferred substrates worldwide for dairy fermentations. The use of raw milk for the production of fermented milk products is declining steadily, but continues in some countries for reasons of tradition. It should be emphasized that the microbiological quality of raw milk is variable and unpredictable. Irrespective of traditions, pasteurized milk together with well characterized starter cultures has proven to eliminate non–spore-forming pathogenic bacteria prior to dairy fermentations.

23.3 SOURCES OF MICROORGANISMS USED IN DAIRY FERMENTATIONS

Analysis of fermented dairy products offered on the market shows that they are developed from products that were originally fermented spontaneously and are now manufactured by using well characterized starter cultures. These cultures have been isolated and developed from the traditional spontaneously fermented products in the course of the last hundred years.[18]

23.3.1 Taxonomy and Identification

Following the initial isolation of starter culture components from spontaneously fermented products like sour cream, cheeses, and yogurt by pioneers (Weigmann, Storch, and Cohn), Orla-Jensen[102] was the first bacteriologist to propose a clear differentiation scheme for lactic acid bacteria on the basis of morphology (rod/coccus), sensitivity to oxygen, presence of catalase, gas formation (hetero- versus homofermentative bacteria), and response to growth temperature (mesophilic versus thermophilic bacteria). However, these properties were not sufficient to differentiate clearly all occurring lactic acid bacteria, propionibacteria, bifidobacteria, brevibacteria, and closely related forms as well as yeasts and molds found in and on fermented dairy products. It was due to modern methods of biochemistry, chemotaxonomy (cell wall composition, fatty acid and protein profiles), and molecular biology (DNA-DNA hybridization, ribosomal RNA sequences) that a very clear picture of the evolutionary relations and finally a clear taxonomy evolved.[120] The main organisms recognized as important in starter cultures are shown in Table 23-3. Only a limited number of bacterial, yeast, and mold genera are important for dairy starter cultures. Some of the species used are only found in dairy products, which means that they have been selected and cultivated empirically by the dairy manufacturers in the course of hundreds or thousands of years of practical use.

The classical whey culture (ie, whey remaining from the previous fermentation was inoculated into the milk for the next production) that was therefore a very successful method of propagating the microflora adapted to the corresponding product and environment without knowing the exact composition. The most important consequence of the modern clear-cut taxonomy is the fact that the microorganisms used in starter cultures are very well characterized and differentiated from related pathogenic or undesirable microorganisms (eg, from *Listeria*, enterococci, hemolytic streptococci, hemolytic staphylococci).[125] Figure 23-1 shows an evolutionary tree based on 16S ribosomal RNA sequences providing clear-cut positions for the bacteria used in dairy fermentations as compiled in Table 23-3. It should be mentioned that specific nucleotide probes are available to identify pathogenic bacteria and starter bacteria by PCR techniques or colony hybridization.[73,80]

Table 23-3 Main Microorganisms Used in Starter Cultures for Fermented Dairy Products

Species	*Normal Habitat*	*Function*
Brevibacterium linens	cheese surface	1. surface ripening 2. competitive displacement of other microbes
Lactobacillus helveticus	Swiss cheese, yogurt	1. thermophilic lactic fermentation 2. slow protein degradation during ripening
Lactococcus lactis subsp. *lactis* subsp. *cremoris* subsp. *diacetylactis*	sour milk cheddar cheese Gouda cheese fresh cheese	1. mesophilic lactic fermentation 2. diacetyl production 3. slow proteolysis
Leuconostoc lactis	sour milk and butter cultures	aroma formation (diacetyl)
Propionibacterium spp.	Swiss cheese	1. propionic fermentation 2. eye formation
Streptococcus thermophilus	yogurt culture	lactic fermentation
Candida kefir	kefir	alcoholic fermentation
Penicillium camemberti	Camembert cheese	1. surface ripening 2. competitive displacement of undesired molds
Penicillium roqueforti	Roquefort cheese bread	aroma formation (methylketones)
Bifidobacterium spp.	intestine	"probiotic" action
Lactobacillus acidophilus	intestine, vagina	"probiotic" action

Source: Adapted with permission from M. Teuber, A. Geis, U. Krusch, and J. Lembke, Biotechnologische Verfahren zur Herstellung von Lebensmitteln und Futtermitteln, in *Handbuch der Biotechnologie*, P. Prave, U. Faust, W. Sittig, D.A. Kukatsch, hrsg, Olderbourg Verlag Munchen, 4, Aufl. pp. 479–540, © 1994, R. Oldenbourg Verlag Munchen-Uiew.

23.3.2 Brief Microbiological Characterization of Starter Microorganisms and Important Contaminant Microbes (Figure 23-2)[109,117,132,147]

Bifidobacterium[7]

These bacteria are typical of the human intestinal tract and are strictly anaerobes belonging to the family of *Actinomycetes*. Bifidobacteria colonize the originally sterile intestines of breast-fed infants within the first week of life, resulting in up to 10^{11} colony-forming units (cfu) per g of feces. The low pH (about 4) of the infant's stools results from a typical heterofermentative lactic acid fermentation, during which fructose-6-phosphate is converted by a specific phosphoketolase into erythrose-4-phosphate and acetyl-phosphate, because the normal pathway through the pentose-phosphate cycle is blocked due to the unavailability of the glucose-6-phosphate dehydrogenase. Thus, two molecules of fructose-6 P give rise to two molecules of L(+) lactic acid and three molecules of acetic acid. Bifidobacteria can be also found in high numbers in the intestines of adults. The acids produced by bifidobacteria are thought to play an important role in the control of putrefactive microorganisms in the bowel. Outside the intestines the bifidobacteria die quickly in the presence of traces of atmospheric oxygen. They grow well under anaerobic conditions in milk but the flavor changes thus produced are largely undesirable (predominantly acetic acid!). Research into the pos-

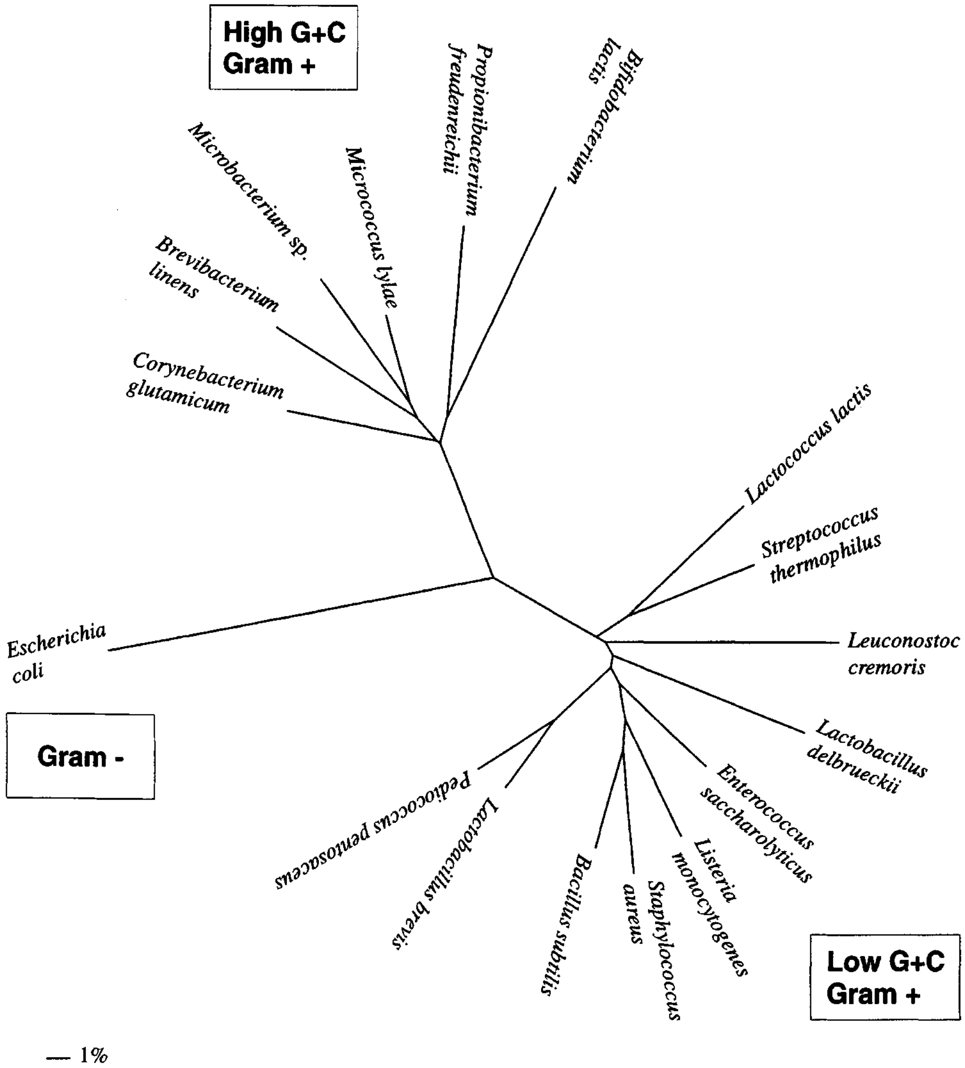

Figure 23-1 Phylogenetic Tree of Desirable and Undesirable Gram-Positive Bacteria Important for Milk Fermentations. The tree was constructed by the neighbor-joining technique on the basis of 16S ribosomal RNA sequences available in public databanks (eg, GenEMBL) according to the principle of Schleifer and Ludwig.[120] Courtesy of G. Dasen, Eidgenossische Technische Hochschule, Zurich, Switzerland.

sibility of introducing bifidobacteria into the intestinal flora by means of fermented dairy products shows inconclusive results (Section 23.6).

Brevibacterium linens

These bacteria are responsible for the surface-ripening of red-smear and yellow-smear cheeses. *B. linens* is Gram-positive, rod-shaped, and obligately aerobic and belongs to the high G+C branch of bacteria (Figure 23-1). The piquant aroma produced by *B. linens* is due to its strongly proteolytic and lipolytic activities.

Coryneform Bacteria[140]

Gram-positive strictly aerobic bacteria like *Arthrobacter/Micrococcus* and *Microbacterium* species from the high G+C branch are part of cheese surface microflora and contribute to ripening in a similar way to *Brevibacterium linens.* A full description of their function still needs considerable research efforts.

Enterococcus[32]

Bacteria in this genus include the species *E. faecalis* and *E. faecium,* which are important to the

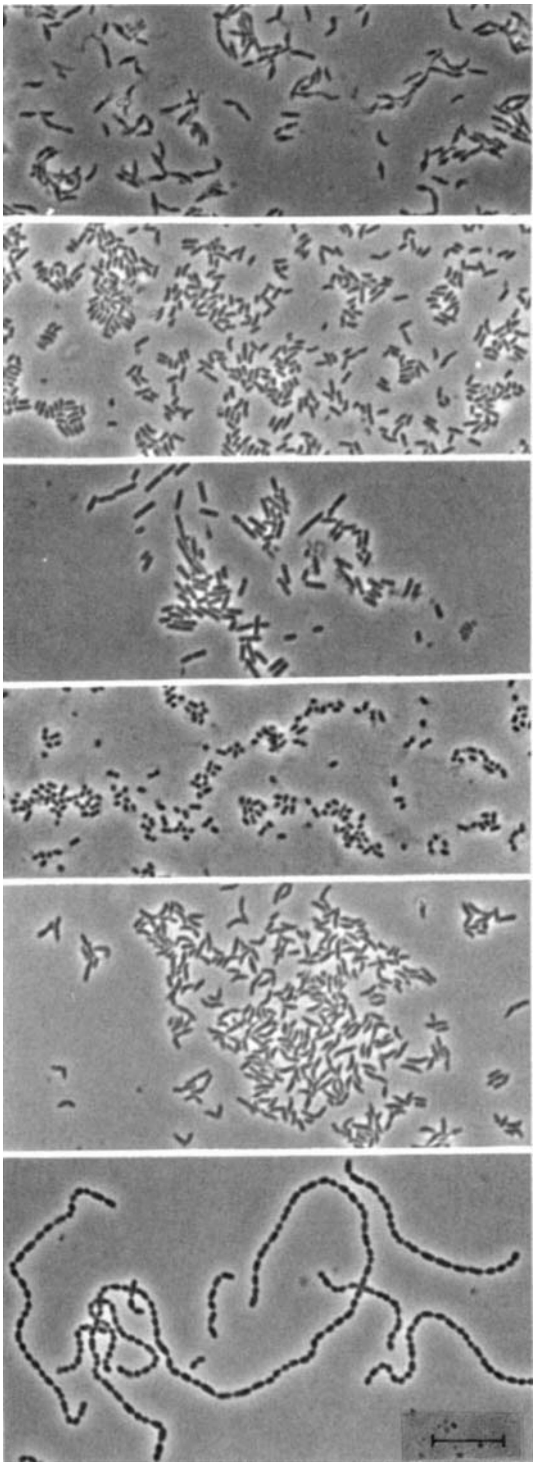

Figure 23-2 Phase Contrast Photomicrograph of Important Bacteria for Dairy Fermentations, from Top to Bottom: *Bifidobacterium bifidum, Brevibacterium linens, Lactobacillus helveticus, Lactococcus lactis, Propionibacterium shermannii, Streptococcus thermophilus.* Courtesy of B. Kollöffel and U. Schuler-Schmid, Eidgenössische Technische Hochschule, Zurich, Switzerland.

dairy industry. Originating in intestinal tracts of many animals, these bacteria are relatively thermoresistant, ie, they can survive pasteurization conditions in small numbers ($D_{65°C}$ values around 2 minutes, z-values around 7 to 8).[91] Subsequently, under suitable growth conditions, they can multiply to high counts. Because they are highly osmo-tolerant, they may cause problems especially in concentrated dairy products such as evaporated milk and whey concentrates, as well as in cheese. These bacteria are found regularly in raw milk, but only under nonhygienic manufacturing conditions are they found in cheese from pasteurized milk. Enterococci are the causative agents of certain infectious diseases, including urinary tract inflammation or endocarditis.[63] Food poisoning due to enterococci has not yet been conclusively documented, as experiments with volunteers have not shown any poisoning symptoms. For certain very highly salted fresh and rennet-coagulated cheese, the use of starter cultures with enterococci has been suggested. However, as the manufacture of these products is possible using unquestionably nonpathogenic lactic acid bacteria, the use of enterococci as components of starter cultures should be rejected, particularly if they carry transmissible antibiotic-resistant genes (Section 23.7.4). Recently, cultures of *E. faecium* have been promoted as a probiotic component of animal feeds or even yogurt for human consumption, aiming at a possible colonization of the individual's intestine to act as an inhibitor of growth of pathogenic enterobacteria such as *Salmonella.* As with the colonization of human intestines by bifidobacteria, the effectiveness of the probiotic use of *E. faecium* is very difficult to confirm experimentally (see Section 23.6).

Lactic Acid Bacteria[132]

These are Gram-positive, rod- or coccoid-shaped bacteria whose main metabolic product resulting from breakdown of sugars is lactic acid. According to this definition, this group also contains a number of undesirable or pathogenic organisms such as enterococci, hemolytic streptococci, and pneumococci. In food technology, the term identifies those organisms that are used in various food- or feed-fermentation processes. According to modern developments in bacterial taxonomy, all technologically useful lactic acid bacteria are classified within the genera *Streptococcus, Lactobacillus, Lactococcus, Leuconostoc,* and *Pediococcus.* In practical aspects of the fermentation processes, the technological properties play a more important role for the differentiation and determination of these bacteria than the molecular biology approach. All lactic bacteria have an essentially anaerobic metabolism, although they may not be inhibited by the presence of atmospheric oxygen. Thus, they are occasionally described as being microaerophilic. Technologically, the following are some important differentiation characteristics:

1. **Homofermentative versus heterofermentative metabolism (lactic acid metabolism).** Homofermentative lactic acid bacteria used in dairy technology include all *Lactococcus* species, *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and subsp. *lactis*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Pediococcus* species. Heterofermentative lactic acid bacteria in the physiological sense include all *Leuconostoc* species, *Lactobacillus kefir*, *Lactobacillus brevis*, *Lactobacillus fermentum*, and *Bifidobacterium bifidum*.
2. **Temperature response.** Thermophilic lactic acid bacteria include *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and subsp. *lactis*, and *Lactobacillus helveticus*. Mesophiles include all *Lactococcus*, *Leuconostoc* and *Pediococcus* species, *Lactobacillus casei*, and *Lactobacillus kefir*.
3. **Salt tolerance.** This is especially high (over 6.5% NaCl) for some *Enterococcus* and *Pediococcus* species, moderate for some *Lactobacillus* species (eg, *L. casei*), and low for all *Lactococcus* species.
4. **Production of lactic acid stereoisomers.** These are predominantly (>95%) L(+) lactate in all *Lactococcus* and *Streptococcus* species, *Lactobacillus casei*, and *Bifidobacterium bifidum*, predominantly D(–) lactate in *Lactobacillus delbrueckii* subsp. *lactis*, and *Leuconostoc*. A mixture of L(+) and D(–) lactate (racemate) is produced by *Lactobacillus acidophilus*, *Lactobacillus helveticus*, and *Pediococcus*.

The earlier differentiation of the lactobacilli into thermobacteria (*Lactobacillus delbrueckii, lactis, helveticus, acidophilus*), streptobacteria (*L. casei, plantarum, curvatus*) and betabacteria (*L. fermentum, brevis, buchneri*) has been largely abandoned today.

Lactic acid bacteria usually have requirements for complex nutrient media; their needs include amino acids, vitamins, trace elements such as manganese, and other growth factors. Suitable media for their propagation include milk, tomato juice, and others. Often they are very sensitive to their own product, lactic acid. If proper pH control is used in the production of commercial starter cultures, unusually high bacterial numbers (about 10^{10} ml^{-1}) can be obtained.

Lactobacillus acidophilus is a lactic acid bacterium whose natural habitat is the intestine and vagina of warm-blooded animals. Occasionally, it may be added to fermented dairy products for its proposed positive effect on the intestinal flora of the consumers. This, however, is very difficult or impossible to prove scientifically (Section 23.6). Presently, these bacteria are considered to be harmless to human health.

Lactobacillus brevis is a heterofermentative lactic acid bacterium, found especially in sour dough (in breadmaking). It is undesirable in fermented dairy products because of uncontrollable gas production.

Lactobacillus bulgaricus, as found in the traditional literature, is now regarded as a subspecies of *Lactobacillus delbrueckii*: *L. delbrueckii* subsp. *bulgaricus*. It is a thermophilic and homofermentative lactic acid bacterium that forms a main component of the traditional yogurt culture.

Lactobacillus casei subsp. *casei* is a homofermentative and mesophilic lactic acid bacterium present particularly in hard cheeses and in cheeses made from raw milk.

Lactobacillus helveticus is a thermophilic, homofermentative lactic acid bacterium that is a principal component of the microflora in hard cheeses.

Lactobacillus kefir is a heterofermentative lactic acid bacterium in kefir grains, to a certain extent related to *Lactobacillus brevis*.

Lactobacillus lactis, in modern taxonomy regarded as a subspecies of *L. delbrueckii*, is a thermophilic, homofermentative lactic acid bacterium. It is a component of starter cultures for hard cheeses.

Lactococcus[134] is a genus of mesophilic lactic acid streptococci (formerly *Streptococcus*) that are present in many starter cultures for cheese, cultured milk, sour cream, and fresh cheese. It includes the subspecies *L. lactis* subsp. *lactis* (used for acid production), *L. lactis* subsp. *cremoris* (used for acid production), and *L. lactis* subsp. *lactis* biovar *diacetylactis* (used for production of the aroma compound diacetyl). The *cremoris* subspecies is associated only with milk and milk products, some of the *lactis* strains have also been found in plant materials. However, these wild strains have different properties from those of strains predominating in milk and dairy products. The strains used in the dairy industry today can be considered as "domesticated" because they have been used for their particular purpose for hundreds of years and have been cultivated unknowingly for a long time. From the health standpoint they are considered to be completely harmless bacteria. *Lactococcus* is homofermentative and produces only L(+) lactic acid. The proteolytic system required for growth in milk is specific for β-casein in many strains. Some strains of *Lactococcus* produce an antibiotic polypeptide, nisin, that is mainly effective against Gram-positive bacteria (eg, clostridia). Many technological functions of *Lactococcus* are coded on plasmids; this explains why today *Lactococcus* is a favorite research subject of molecular engineering.[48]

The *Leuconostoc* group of heterofermentative lactic acid bacteria decompose lactose to D(–) lactic acid. *L. cremoris* is used in butter culture for the formation of aroma (diacetyl).[26]

Propionic Acid Bacteria[20]

These bacteria are used in production of some cheese types for propionic acid fermentation. *Propionibacterium* induces conversion of lactic acid to propionic acid under anaerobic conditions. This biochemically very complex reaction results in conversion of three molecules of lactic acid into two molecules of propionic acid, one molecule of acetic acid, one molecule of carbon dioxide, and one molecule of water. The propionic acid bacteria can be found in the rumen microflora and intestinal tract of ruminants. They belong to the *Actinobacteria* class and are not strictly anaerobic but microaerotolerant. The carbon dioxide produced during the propionic fermentation causes the eye formation in Swiss cheese. The maximum amount of propionic acid in such cheeses is about 100 mMol kg^{-1} cheese. The optimum reaction temperature is well above 20°C, corresponding to the conditions where it occurs naturally (rumen and intestines of ruminants). For controlled eye formation in Emmental cheese, temperatures of about 24°C are used. This process takes about 12 weeks. Propionic acid bacteria are sensitive to nitrate. Thus, in cheeses in which propionic fermentation is desired, late-blowing through growth of butyric acid bacteria cannot be controlled by the addition of nitrate. Propionic acid bacteria are available commercially as a liquid starter culture.

Penicillium roqueforti[118]

This is a blue mold used for the production of Roquefort, Gorgonzola, Stilton, and other blue cheese varieties. It is found widely in nature, eg, on moldy grain or bread; however, these wild strains are not suitable as starter cultures for cheese manufacture. The strains used by the dairy industry have been in use for several decades and are well characterized both microbiologically and technologically. Starter cultures, traditionally produced on sterilized bread and washed off with physiological saline solution, are available in both liquid and dried form. They contain about 10^9–10^{10} conidia g^{-1} of preparation and are added to the cheese milk at the beginning of the coagulation period. To ensure mold growth in the inner areas of the cheese, the cheese loaf must be pierced to allow the entry of the required oxygen. *P. roqueforti* strains are proteolytic and lipolytic and the resulting characteristic aroma of the blue cheese is correspondingly complex. The main factors appear to be the degradation products of fatty acid breakdown, including methyl ketones such as 2-heptanone, 2-nonanone, 2-pentanone, and 2-undecanone, as well as the corresponding alcohols 2-pentanol, 2-heptanol, and 2-nonanol, together with the usual products of protein degradation. Because *P. roqueforti* can easily be confused with undesirable contaminant molds, the culture production requires stringent and continuous control. The strains used by the dairy industry produce in the cheese up to 7 ppm of roquefortin and a few strains up to 15 ppm mycophenolic acid.[38] These metabolites are considered to be of no significance to public health in the amounts ingested. Carcinogenic mycotoxins have not been detected in these cheeses. Animal experiments carried out by the German Center for Cancer Research have not established any risk associated with the consumption of blue cheese.[103]

Penicillium camemberti[118]

This is used for the surface-ripening of Camembert, Brie, Handkäse, and other cheeses. The nomenclature of the *Penicillia* has undergone some confusing changes in recent years. From the standpoint of practical use the situation can be summarized as follows: All "white molds" important for dairy technology belong to the original species *P. camemberti.* These old strains are characterized by a gray to bluish appearance of their well-developed mycelia. Conversely, strains with pure white mycelium were first classified as *P. candidum* and later reclassified as *P. caseicolum*. Thus, the gray-blue mold should be denoted as *P. camemberti*, the pure white mold as *P. caseicolum*, while the name *P. candidum* is still often used correctly as a synonym for *P. caseicolum*. Production of starter cultures of *P. camemberti* takes about 4 weeks using a semi-solid nutrient medium. The conidia developed in the process are washed with physiological saline solution, and adjusted to concentrations of about 10^9–10^{10} germination-ready conidia per g preparation for commercial use. Such cultures can be kept in a refrigerator for several months. Freeze-dried conidia are also available now; however, the proportion of nonviable conidia in the powdered form is much higher (up to 90%) than in the liquid preparations of equal concentration. If regular production of uniform quality culture combined with prompt delivery service can be provided by the manufacturer, it is easier to utilize the liquid culture because there are no problems in dissolving it in the cheese milk. Being an aerobic organism, *P. camemberti* develops only on the cheese surface. The mycelium shows a rapid growth about 5 days after the cheese production. A uniform growth of the inoculum mold inhibits any undesirable contaminating molds. Preparations known as "anti-*Mucor* cultures" show this property in the laboratory but the effect can seldom be seen under practical conditions. All 62 *P. camemberti* strains tested that are currently being used for cheese production have been shown to produce cyclopiazonic acid, a substance with hepato- and neurotoxic effects for rats (the LD_{50} for these animals is between 36–63 mg kg^{-1} body weight). In the normally ripened and handled cheese, the maximal levels of cyclopiazonic acid were 0.3 ppm and the average was about 0.15 ppm.[38] The average consumption of Camembert cheese in the Federal Republic of Germany results in uptake of about 1 ng kg^{-1} body weight. Thus, from the public health standpoint, the consumption of cyclopiazonic acid in Camembert and Brie cheeses is of no concern. Carcinogenic mycotoxins such as aflatoxin, ochratoxin, and sterigmatocystin are not produced.

Recently, submerged fermentation of *P. roqueforti* and *P. camemberti* has been successfully developed instead of solid substrate fermentation of starter culture preparations[11] (Figure 23-3).

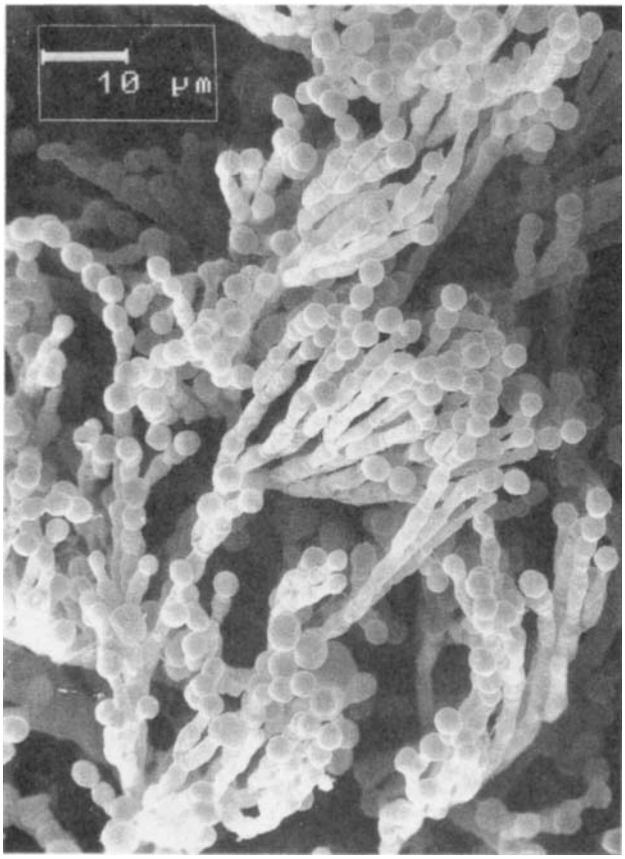

Figure 23-3 Scanning Electron Micrograph of *Penicillium camemberti* Grown in Submerged Fermentation, a New Method To Produce Conidia for Dairy Starter Cultures. Courtesy of H. Neve and W. Bockelmann, Federal Dairy Research Centre, Kiel, Germany.

Staphylococcus aureus

All over the world this bacterium is the most frequent cause of mastitis in cattle, sometimes also in sheep and goats (see Chapters 22 and 47). It may be present in up to 70% of samples of raw milk, in numbers up to 10^2 to 10^5 cfu ml^{-1}.[29,30]

Yeasts

Yeasts are eucaryotic microorganisms belonging to fungi, often regarded as budding fungi. In contrast to most other fungi, yeasts are, as a rule, single-cell organisms that can switch between aerobic and anaerobic metabolism. The importance of yeasts for the dairy industry lies primarily in their acid tolerance and ability to metabolize lactic acid, resulting in spoilage of fermented dairy products. As starter cultures they are used to a limited extent in the manufacture of kefir and in the ripening of red- and yellow-smear cheeses where they cause primary neutralization of the cheese surface enabling the subsequent growth of the smear bacteria (mainly *Brevibacterium linens*).

Raw milk regularly contains various yeasts, including (in order of abundance): *Geotrichum candidum, Trichosporon cutaneum, Candida curvata, Candida famata, Candida globosa, Kluyveromyces marxianus, Candida kefir, Candida lambica, Candida krusei,* and *Hansenula anomala.* During pasteurization, all these are inactivated.[35]

The shelf life of fresh cheese, quark, and cottage cheese is limited primarily by the presence of yeasts. One yeast cell in a 250 g package can multiply in the cold storage conditions to more than 1 million cells

g^{-1} product within 2 to 3 weeks. Because yeasts can produce a noticeable off-flavor (defect: "yeasty" or "fermented-fruity") with 10^4–10^6 cells g^{-1} (depending on the species), packages with such high yeast counts are considered spoiled in the sense of the Food Act. In addition to the sensory effects, the yeast fermentation leads to production of alcohol and carbon dioxide and to swelling or blowing of the containers. Yeasts do not produce any mycotoxins, so that the consumption of yeast-contaminated products is not dangerous from the health standpoint. The most abundant yeasts in fresh cheese (in order of quantity) are *Geotrichum candidum, Kluyveromyces marxianus, Candida valida, Candida kefir, Pichia membranaefaciens, Candida lipolytica,* and *Candida famata.* The presence of yeasts in fresh cheese, quark, and similar products can be avoided only by ensuring yeast-free fermentation followed by recontamination-free aseptic packaging, or by pasteurization and aseptic packaging after fermentation.[36]

Lactose-fermenting yeasts, especially *Candida kefir* and *Kluyveromyces marxianus* subsp. *marxianus,* are natural components of the classical kefir grains. In addition, lactose-negative yeasts such as *Saccharomyces cerevisiae* and *S. unisporus* as well as *Candida holmii* are often present; these can utilize the breakdown products of lactose (glucose and galactose). In classical kefir, these yeasts can produce up to 1% ethanol. The accompanying production of carbon dioxide causes container swelling if unsuitable packaging is used. In a product denoted as "mild kefir" in the Federal Republic of Germany, the lactose-fermenting yeasts may be omitted from the starter culture for this reason.[37]

23.3.3 Biochemistry and Genetics of Starter Cultures

A thorough knowledge of their biochemical pathways and the genetics regulating these pathways is a prerequisite to a controlled application of starter cultures in modern dairy technology. The advance of genetic engineering has lead to an almost complete elucidation of the transformation from lactose to lactic acid by homofermentative bacteria, and to lactic acid, acetic acid, ethanol, and CO_2 in heterofermentative bacteria[68] and characterization of the enzymes involved. Likewise, the mechanisms of the breakdown of casein during milk fermentation and cheese ripening have been investigated in great detail.[72]

The formation of the important aroma compound diacetyl from citrate by specific bacteria (*Leuconostoc* spp., *Lactococcus lactis* subspecies *lactis* biovar *diacetylactis*) is well understood.[57]

The scientific progress is especially evident in the genus *Lactococcus* because most of the technologically important functions are coded for on plasmids, which are more easily accessible to genetic analysis and genetic engineering than enzymes coded for on the bacterial chromosome. Because elaborate reviews are available,[48,134] it suffices to mention the main plasmid-coded traits: lactose transport and metabolism, casein degradation by cell-wall proteases, citrate and oligopeptide transport by specific permeases, bacteriophage protection by restriction/modification and abortive infection, formation of extracellular polysaccharides, bacteriocin production and immunity, insertion (IS) element–dependent recombination and cointegrate formation, antibiotic resistances, heavy metal and UV resistance, conjugal transfer and mobilization of plasmids, and plasmid replication.

Based on small cryptic plasmids, cloning, expression, and shuttle vectors have been developed. Cloning and expression of homologous and heterologous genes as well as secretion of the gene products have been successfully achieved. To become independent of antibiotic-resistant selection markers, food-grade selection markers have been introduced such as the lacF marker present in pNZ1125 used to express the pepN gene (a debittering peptidase with potential for accelerated cheese ripening). However, to the author's knowledge, genetically modified lactic acid bacteria are still not applied in the industry because of safety and legal concerns.

The main biochemical and ecologically important properties of some dairy lactic acid bacteria are listed in Table 23-4. As mentioned earlier, once curd formation by lactic acid fermentation or by action of rennet has taken place, the activity of the coagulum-embedded microorganism can only be manipulated by different temperature regimes, diffusion of salt from the brine into the cheese interior, and by loss of moisture during cheese ripening. In addition to the temperature and salt tolerance data, Table 23-4 lists the important metabolic products and their concentrations in fermented milk. Biochemically important aspects of propionibacteria, bifidobacteria, yeasts, and molds are mentioned in the paragraphs on specific products.

23.3.4 Propagation and Preservation of Starter Cultures[18,119,136]

Starter cultures are needed and applied by the industry producing food and feed as indicated in Table 23-3. Dairy products are almost always made with

Table 23-4 Ecologically Important Properties of Some Dairy Lactic Acid Bacteria

Species	*Optimum Growth Temperature (range)*	*Heat Tolerance 60°C/ 30 minutes*	*Lactic Acid in Milk (%)*	*Latic Acid Isomer*	*CO_2 Production*	*Proteolytic Activity*	*Lipolytic Activity*	*Citrate Fermentation*	*Flavor and Aroma Compounds*	*Acetic Acid (%)*	*Alcohol*	*H_2O_2 Production*	*Salt Tolerance (% max.)*
Mesophilic species													
Lactococcus lactis subsp. *lactis*	28–31 (8–40)	±	0.8–1.0	L(+)	–	+	±	–	+	–	±	+	4.0–6.5
L. lactis subsp. *cremoris*	22 (8–39)	±	0.8–1.0	L(+)	–	+	±	–	+	–	±	+	4.0
L. lactis susp. *lactis* biovar *diacetylactis*	28 (8–40)	±	0.8–1.0	L(+)	+	+	±	+	+++ diacetyl	–	±	+	4.0–6.5
Leuconostoc mesenteroides subsp. *cremoris*	20–25 (4–37)	–	0.1–0.3	D(–)	±	±	±	+	+++ diacetyl	0.2–0.4	±	±	6.5
Thermophilic species													
Streptococcus thermophilus	40–45 (20–50)	++	0.7–0.8	L(+)	–	±	±	–	++ acetaldehyde	trace	–	±	2.0
Lactobacillus delbrueckii subsp. *bulgaricus*	40–45 (22–52)	+	1.5–4.0	D(–)	–	+	±	–	++	trace	trace	+	2.0
L. delbrueckii subsp. *lactis*	40–45 (22–52)	+	1.5–4.0	D(–)	–	+	±	–	+	trace	trace	+	2.0
Lactobacillus acidophilus	37 (20–48)	–	0.3–2.0	DL	–	±	±	–	+	+	trace	+	6.5
Lactobacillus casei subsp. *casei*	37 (15–45)	–	1.2–1.5	L(+)	–	±	±	–	±	+	trace	+	2.0

Source: Adapted with permission from R.C. Chandan and K.M. Shahani, Other Fermented Dairy Products, in *Biotechnology*, Vol. 9, 2nd ed., H.R. Rehm, G.A. Reed, A. Puhler, and P. Stadler, eds., pp. 386–418, © 1995, Wiley-VCH.

commercial cultures. Because of its advanced state, the manufacture of lactococcal dairy cultures is described, but the principles are the same for all lactic acid bacteria.

Liquid, dried, and frozen starter cultures are in use. Starter cultures must have a high survival rate of microorganims coupled with optimum activity for the desired technological performance, eg, the fermentation of lactose to lactate, controlled proteolysis of casein, and production of aroma compounds like diacetyl. Because the genes for lactose and citrate fermentation as well as for certain proteases are located on plasmids, continuous culture has not been successful because fermentation-defective variants develop easily. In most instances, pasteurized or sterilized skim milk is the basic nutrient medium for the large-scale production of starter cultures because it ensures that only lactococci fully adapted to the complex substrate, milk, will develop. For liquid starter cultures, the basic milk medium may be supplemented with yeast extract, glucose, lactose, and calcium carbonate. To obtain optimum activity and survival, it may be necessary to neutralize the lactic acid that is produced by addition of sodium or ammonium hydroxide. Because many strains of lactic streptococci produce hydrogen peroxide during growth under microaerophilic conditions, it has been beneficial to add catalase to the growth medium, thus leading to cell densities of more than 10^{10} viable units ml^{-1} of culture.

For the preparation of concentrated starters, the media are clarified by proteolytic digestion of skim milk with papain or bacterial enzymes to avoid precipitation of casein in the separator used to collect the lactococcal biomass. The available self-cleaning clarifiers, eg, bactofuges, concentrate the fermentation broth to cell concentrations of about 10^{12} cfu ml^{-1}. These concentrates can either be lyophilized or are preferentially transferred dropwise into liquid nitrogen. The formed pellets are packed in metal cans or cartons and are kept and shipped at −70°C (dry ice). These modern starter preparations allow the direct inoculation of cheese vats, because the bacteria immediately resume logarithmic growth if harvested in the late logarithmic growth phase. This modern technology shifts most of the microbiological work and responsibility from the cheese factory to the starter producer.

The classical liquid starter culture with about 10^9 cfu ml^{-1} and traditional lyophilized cultures containing about 10^9 cfu g^{-1} must be further propagated in the factory. However, lyophilized cultures can be transported easily and kept at ambient temperature for several months. Concentrated lyophilized starters are also suited for direct vat inoculation having a short lag phase, however, before growth is resumed. Direct vat inoculation may have a certain advantage regarding protection against bacteriophages, which are common in the open dairy fermentation systems (see below).

23.3.5 Commercially Available Starter Cultures[18]

(Consult also catalogs of starter culture producers: Texel, Marshall-Miles, Christian Hansen, and Labor Wiesby.)

Several starter culture producers are active in the field of dairy fermentations. In modern dairying, provision of cultures has almost completely switched from "natural" whey cultures to industrially produced ones. The culture industry offers a variety of cultures to the dairy industry. In culture catalogs, the available cultures are divided into the following categories:

1. Mesophilic cultures of lactic acid bacteria
2. Thermophilic cultures of lactic acid bacteria
3. Molds (*P. camemberti, P. roqueforti*)
4. Yeasts (*Geotrichum candidum, Candida, Brettanomyces*, etc.)
5. Special cultures (propionibacteria, *Brevibacterium linens*)
6. Probiotic cultures (bifidobacteria, *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus casei* subsp. *rhamnosus*, etc.)

The performance of all starter cultures has to be well characterized. For citrate-fermenting mesophilic cultures (*Lactococcus* and *Leuconostoc* species) these characteristics include the time to reach pH 4.5 (about 20 hours), acid and aroma production, gas formation, acidification capacity (maximum pH after 48 hours at 20°C about 4.3 to 4.4), activity of the bulk starter culture (pH value after 6 hours fermentation at 30°C, about 4.8 to 5.15 at 1% inoculation), and recommended maximum cooking temperatures (38° to 39°C). If gas formation has to be slow to guarantee regular eyes, eg, in Gouda cheese, the initial concentration of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc* has to be low.

Mesophilic cultures without citrate fermentation should give pH 4.4 within 18 hours, pH 4.15 to 4.25 within 48 hours at 22°C, and pH 4.8 to 5.0 after 6 hours fermentation at 30°C. If thermophilic cultures are added, acidification capacities (pH values of 3.4 to 4.1 after 48 hours at 22°C) and cooking temperatures (42° to 48°C) are increased.

Thermophilic single-species cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus*, subsp. *lactis*, or *Lactobacillus helveticus*) have acidification capacities that give pH 3.2 to 4.25 after 24 hours. pH values developed are between pH 4.10 after 15 hours at 37°C and pH 4.3 after 6 hours at 42°C. For these cultures, information on proteolytic activities is critical. Very often, polysaccharide (extracellular slime)–producing strains are added to increase viscosity.

Thermophilic *Streptococcus thermophilus* cultures for mild acidification, in contrast, have acidification capacities producing pH 4.3 to 4.65 after 24 hours at 42°C, pH 4.7 to pH 5.3 is obtained after 7 to 9 hours. Polysaccharide-producing strains are of prime importance for yogurt production to aid mechanical stability of the curd without addition of external stabilizers. Fast *S. thermophilus* cultures reach pH 4.4 to 4.6 within 6 hours of fermentation.

Cultures of multiple, thermophilic species contain a mixture of *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* for fast and strong acid production. pH 4.5 is obtained within 3 hours, the overall acidification capacities are around pH 3.40 to 3.65 after 24 hours at 42°C. After 2 hours at 42°C, pH values of 4.95 to 5.05 are obtained.

Thermophilic cultures for mild acidification (4.5 to 7 hours to obtain pH 4.5) contain *S. thermophilus* and *Lactobacillus acidophilus*. 24-hours fermentation capacities at 42°C are around 4.0 to 4.05. Additional species used in such cultures are *Lactobacillus delbrueckii* subsp. *bulgaricus* and *lactis* (slow, protease-weak strain) and several bifidobacteria. *Penicillium roqueforti* is available as semifast and fast cultures with piquant aroma potential, differing proteolytic and lipolytic capacities, and in color shades white, light blue-green, light gray-green, blue green, gray-green, and intensive dark blue-green. Ripening times with these cultures vary between 5 and 7 weeks. In addition, water exudation from the product and production of bitter taste are variable properties.

Penicillium candidum is available as fast or semifast culture with different macroscopic properties of the cheese surface mycelium: very white, medium high, normal density to cream colored, high mycelium, and very high density mycelium.

By definition, *Penicillium camemberti* has a blue-gray color. The available yeast cultures include *Debaryomyces hansenii, Candida utilis, Saccharomyces cerevisiae,* and *Mycoderma* species. Brevibacteria may be orange, yellow-orange, or cream colored cultures.

Propionibacterium freudenreichii cultures are available with different gas formation capacities. They have to be able to survive the cooking temperatures in Emmental manufacture (up to 55°C).

Pictures of some starter bacteria are shown in Figure 23-2 as they might be seen in a normal microbiological laboratory. They demonstrate that the rather unspectacular morphology is not an obvious criterium to identify these bacteria needing the mentioned physiological and molecular properties in addition.

A production scheme for modern bacterial starter cultures is outlined in Figure 23-4.

23.3.6 Quality Standards for Dairy Starter Cultures

(See also microbiological specifications in the catalogs of starter culture producers.)

It is usual to provide information on the performance of starter cultures under practical manufacturing conditions. Some of the important properties have been outlined in the previous section. In practice, a detailed technical information sheet is available for each culture, including information on bacteriophage types. The offered starter cultures must not contain pathogenic or undesirable spoilage microorganisms.

An example for freeze-dried or deep-frozen concentrated cultures suitable for direct inoculation of fermentation vats is given in Table 23-5. The high-risk pathogens *Listeria monocytogenes* and *Salmonella* species (Section 23.5) must be absent in 25 g; coagulase-positive staphylococci must not be detected in 10 g; coliforms should not be present in 1 g; and spoilage microorganisms like yeasts, molds, and enterococci, or undesirable gas-forming organisms, should be restricted to low numbers, which will not grow up to critical levels during fermentation. Freeze-dried or frozen cultures for the inoculation of bulk starter tanks may have stricter limits regarding yeasts, molds, enterococci, staphylococci, and also limits for sulfite-reducing anaerobes (clostridia; not detectable in 1 g) and mesophilic aerobic counts (less than 100 for frozen, and less than 1,000 for freeze-dried starter cultures g^{-1}).

23.3.7 Natural (Artisanal) Starter Cultures[81]

Some natural whey cultures have survived from traditional local cheese making into the age of modern microbiology, enabling an analysis of their composition. Table 23-6 gives examples from Italy and Switzerland. The natural culture listed in Table 23-6 is characterized by lactic acid bacteria (*S. thermophilus*, enterococci), which survive a 15 minute heating of raw milk to 65°C with subsequent incubation at 45°C. The other cultures described are characterized by the presence of heterofermentative lactobacilli (*Lactobacillus fermentum*) in addition to the main three thermophilic lactobacilli (*L. helveticus, L. delbrueckii* subsp. *bulgaricus* and

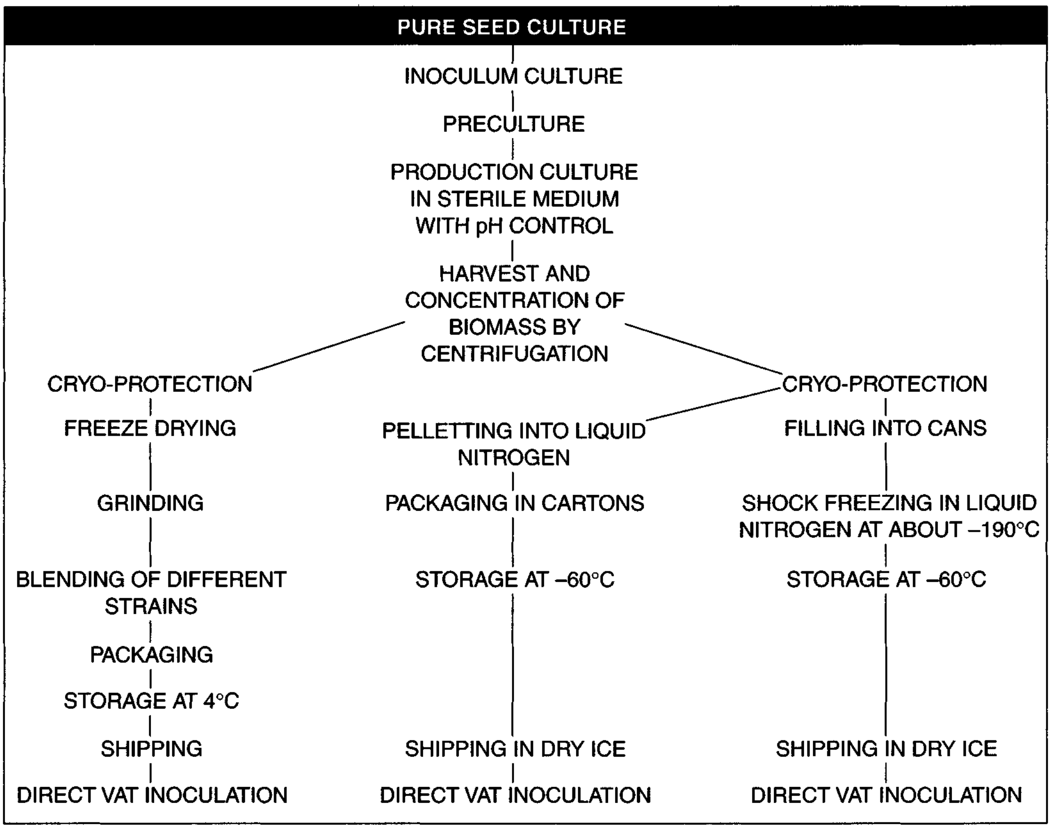

Figure 23-4 Schematic Outline for the Industrial Production of Modern Freeze-Dried, Deep-Frozen, Concentrated Starter Culture Preparations (for more details and references, see Teuber, 1993,[132] and Cogan and Accolas, 1996[18]).

lactis). Table 23-7 gives a detailed microbiological analysis of a natural whey culture used for the fermentation of buffalo milk to mozzarella in southern Italy.[19] This culture is an extremely diversified mixture of thermophilic and mesophilic lactic acid bacteria (streptococci, lactococci, and lactobacilli). A characteristic of this culture is the significant presence of at least 5% of the viable counts as enterococci (*E. faecalis* and *E. faecium*). Without reasonable doubt, enterococci in such cultures (and in raw milk yogurt and cheeses) originate from fecal contamination during milking, in contrast to differing recent explanations, which are not based on experimental data.

23.4 FERMENTED MILK PRODUCTS[16,34,136]

23.4.1 Outline of the Microbiological, Biochemical, and Hygienic Complexity

Before the invention of milk drying, pasteurization, UHT treatment, and sterilization, fermentations of milk were the only ways to conserve the nutritionally important milk ingredients, protein and fat. From the data given in Exhibit 23-1, it is clear that, at present, fermentation processes are probably used for only about one-third of the world production of cow's milk. The cheese production of about 15 million tons requires about 150 million tons of milk. The proportion used for cheese production may be higher in the case of goats' and sheep's milk. The fermentation of milk (eg, cow's, goat's) with defined starter cultures is still a very complex process because of the following conditions:

1. The substrate milk contains truly dissolved compounds like lactose, globular whey proteins, amino acids, vitamins, some minerals, as well as the casein micelles (50 to 300 nm in size) in addition to fat globules surrounded by a membrane composed of phospholipids and proteins.
2. During unstirred fermentation and production of lactic acid, the system changes from a liquid into a semisolid (coagulated milk) or solid (cheese) state. As a consequence, the microorganisms that are initially mobile become immobilized during fermentation.

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Table 23-5 Microbial Quality Standards for Dairy Starter Cultures

Microorganisms	*Standard*	*Media and Incubation*[a]
Total coliforms	n.d. in 1 g	IDF 73 A: 1985
Enterococci	less than 20 in 1 g	Enterococcosel 48 h at 37°C
Yeasts and molds	n.d. in 1 g	IDF 94 A : 1985
Coagulase-positive staphylococci	n.d. in 10 g	IDF 138 : 1990
Listeria	n.d. in 25 g	IDF 143 : 1990
Salmonella spp.	n.d. in 25 g	IDF 93 A : 1985
Leuconostoc (in mixed cultures without *Leuconostoc*)	less than 10 in 1 g	Mayeux-Sandine-Elliker-Agar

[a] IDF = International Dairy Federation Standards No. and year of publication.
Source: Reprinted with permission from Texel, *Dokumentation für die Milch- und Käseindustrie* (Starter Culture Catalogue), © 1992, Rhodia Food SAS.

3. During the fermentation of milk and ripening of cheeses, there are constant changes of the environmental conditions for the microorganisms involved. These include changes in pH, oxygen pressure, and water activity; available nutrients may decrease, and metabolic waste and byproducts (eg, lactic acid, propionic acid, acetic acid, certain amino acids, free fatty acids, biogenic amines, methylketones, diacetyl, formic acid, and benzoic acid) may increase. Within the mesophilic and thermophilic lactic acid bacteria, single strain, multiple strain/species, and undefined strain/species cultures are available enabling changes to be made to avoid susceptibility to bacteriophages. In technological terms, the activities of the culture regarding speed and amount of lactic acid formation, citrate transformation into diacetyl, and CO_2 evolution (necessary for cheese hole development) are part of the culture description.
4. If the final product is pasteurized (as is done for fresh cheeses), a clear definiton of the fermentation endpoint is possible. Cheeses and sour milks not heated after fermentation undergo continuing microbiological and biochemical changes. This should be kept in mind when data on composition are given in this chapter.
5. The times (days to months) and temperatures (6° to 54°) used in production of these foods may vary substantially. These two parameters are the main tools available for controlling the activities of microorganisms fixed inside the solid protein/fat/water matrix of cheeses.
6. The fermentation may be further complicated if a surface microflora develops during cheese ripening in addition to the internal microflora. Under these circumstances, the relative humidity of the atmosphere is of prime importance.

As stated in the introduction, all these tremendously complex fermentation processes have evolved by empirical application. Modern dairy biotechnology tries to imitate the traditional processes and products on a rational and microbiologically well-defined basis.

Two methods of coagulation of milk are used in dairy fermentations: (1) coagulation due to lowering of the pH value as a result of microbial metabolism (sour milk route), and (2) coagulation due to added proteases (chymosin/rennet route). The main fermented dairy products will be described according to this differentiation.

The curdling of milk necessary for the manufacture of sour milk products and cheese is the consequence of a destabilization of the colloidally dissolved casein micelles, which can be achieved biophysically by lowering the pH or biochemically-biophysically by means of the proteolytic enzyme preparation rennet or by a combination of the two processes. Milk protein consists of about 75% to 80% caseins and 15% to 25% whey proteins including lactoglobulin, lactalbumin, serum, and immune globulins. The casein fraction is heat resistant and acid labile, and the whey proteins are denatured by heating but remain in a solution in the critical pH range in dairy products around pH 4.

The caseins are organized into casein micelles between 50 and 300 mm in size and are made up of submicelles. There are about 70×10^{13} casein particles per ml of milk. The micelles of cow's milk contain 42% of α-casein (MW 23,615), 28% of β-casein (MW 23,983), and 14% of κ-casein (MW 19,023), together with 10% of other casein components, 2.9% of calcium, 0.5% of magnesium, 0.8% of organically bound phosphorus, 1.4% of nonbound phosphorus, 0.5% of citric acid, 0.3 of N-acetylneuraminic acid, 0.2% of galactose, and 0.2% of galactosamine. The isoelectric point of the casein micelle is at pH 4.6. The micelle is stabilized by hydrophobic interactions and salt bridges. Based on 1 mole of casein, the micelle contains 24 moles of calcium, 8 moles of organically bound phosphoric acid, 1 mole of inorganic phosphate, and 1 mole of citric acid, and can be regarded as a caseinate-calcium-phosphate-citrate complex. An experimentally substantiated, descriptive model of the casein micelle has been developed by Schmidt.[121]

Table 23-6 Characteristics of Some Natural Starter Cultures Used in Cheese Manufacture in Italy and Switzerland

			Microflora						
Cultures and Relevant Cheese	*Local Name*	*Preparation of Culture*	*Streptococcus thermophilus*	*Lactobacillus helveticus*	*Lactobacillus lactis*	*Lactobacillus bulgaricus*	*Lactobacillus fermentum*	*Lactococci*	*Enterococci*
Natural milk cultures									
Crescenza Italico Mozzarella Others	*Latto-innesto* *Latto-fermento*	Thermization (63°–65°C, 15 min) of raw milk; cooling to 45°C and incubation until a final acidity of 0.4–0.5% lactic acid is obtained (4.5–6 hr)	++						++
Whey cultures									
Water buffalo Mozzarella	*Siero-innesto* *Cizza*	Incubation of whey at the end of cheese making at room temperatures (cooling from 37° to 20°–22°C) until a final acidity of 0.9–1.1% lactic acid is obtained (16–18 hr)	++	++	++	+	+	++	++
Provolone	*Siero-innesto*	Incubation of whey at the end of cheese making at 30°–45°C; final acidity 1.0–1.3% lactic acid	+	++		++	+		
Pecorino Romano	*Scotta-fermento*	Heat treatment of whey at 80°–90°C for 20–30 min to deproteinize; incubation for 24 hr; inoculation of ricotta whey; incubation at 45°C for 24 hr; final acidity 0.7–0.8% lactic acid	+		++	++			
Grana	*Siero-innesto* *Siero-fermento*	Incubation of whey at the end of cheese making at 47°–52°C; final acidity 1.2–1.3% lactic acid	+	++	+	+	+		
Whey culture + rennet									
Emmental Gruyère Sbrinz	Fettsirtenmagen-Lab Présure à la "recruite"	Maceration of dried calf stomachs in whey at 39°–45°C, with or without deproteinization (by boiling at pH 5.0); final acidity 0.9–1.3 lactic acid	++	++	++	+	+		+

Note: Latto-innesto, latto-fermento = milk starter; *siero-innesto, siero-fermento, cizza* = whey starter; *scotta-fermento* = deproteinized whey starter; Fettsirtenmagenlab, Présure à la "recruite" = deproteinized whey starter with rennet.

Source: Reprinted with permission from G.K.Y. Limsowtin, I.B. Powell, and E. Parente, Types of Starters, in *Dairy Starter Cultures*, T.M. Cogan and J.P. Accolas eds., pp. 101–130, © 1996, VCH Publishers.

Table 23-7 Synopsis of Microbial Composition of a Natural Whey Culture for the Production of Mozzarella Cheese from Buffalo Milk in Southern Italy

Species	*Viable Counts (cfu) per ml*
Thermophilic lactobacilli	2×10^8
Lactobacillus delbrueckii ssp. *lactis*	
L. salivarius	
L. helveticus	
Thermophilic streptococci	1×10^8
Streptococcus thermophilus	
Mesophilic lactococci	2×10^8
Lactococcus lactis ssp. *lactis*	
L. lactis ssp. *lactis* biovar *diacetylactis*	
L. lactis ssp. *cremoris*	
Mesophilic lactobacilli	1.6×10^7
Lactobacillus plantarum	
L. casei ssp. *pseudoplantarum*	
Enterococci	3×10^7
Enterococcus faecalis	
E. faecium	

Source: Reprinted with permission from S. Coppola, E. Parente, E.S. Dumontet, and A. La Peccerella, The Microflora of Natural Whey Cultures Utilized as Starters in the Manufacture of Mozzarella Cheese from Waterbuffalo Milk, *Le Lait*, Vol. 68, pp. 295–310, © 1988, Editions Scientifiques et Medicales Elsevier.

Curdling of Milk by Lactic Acid Fermentation

With decreasing pH, the casein micelles lose calcium and phosphoric acid is dissociated. From a pH of 5.2 to 5.3, ie, even before the isoelectric point of 4.6 is reached, the calcium- and phosphorus-deprived micelles begin to precipitate. In this pH region, the coagulum formed occupies the entire original volume of the milk. Only by mechanical agitation (cutting and stirring) and raising the temperature (heating and cooking) does a further aggregation of the casein (syneresis) take place with a consequent excretion of whey from the coagulum.

Curdling of the Milk with Rennet

Rennet is an aqueous extract from the fourth stomach of unweaned calves, which is marketed in aqueous form or as rennet powder, or it may be replaced by chymosin produced by microbial fermentation.[130] The important constituent of rennet is the proteolytic enzyme chymosin (rennin, E.C. 3.4.4.3). Chymosin cleaves the κ-casein molecules very specifically between phenylalanine-105 and methionine-106. In milk, rennet-induced curdling is a two-phase process, which takes place only when sufficient calcium is available. In the absence of calcium, κ-casein is cleaved, but there is no coagulation. In a third phase, during the ripening of the cheese over long periods, other caseins are also hydrolyzed slowly. Pepsin and chymotrypsin act similarly. If cheese is made from raw milk, the milk-derived proteolytic enzyme plasmin contributes considerably to proteolysis during cheese ripening (Table 23-8).

23.4.2 Sour Milk Products[136]

General Aspects

Under suitable conditions (22° to 45°C), the lactic acid bacteria from the very complex microflora present in raw milk[30] are selectively enriched, can multiply at a high rate, and thereby ferment part of the lactose present in the milk to lactic acid. The resulting decrease of the pH and the redox potential suppress many of the other species of microorganisms of the original milk flora and, in particular, the causative agents of spoilage and disease, such as staphylococci, enterobacteria, and clostridia without, however, killing them. This lactic acid fermentation produces from milk a keepable, pleasant-tasting, and highly nutritious product.

In various regions of the world different practices for milk treatment have developed on the basis of the existing climatic conditions. Depending on the type of milk used, products arise such as curdled (sour) milk in Europe, yogurt in the Balkans, kefir in the

Exhibit 23-1 Main Global Dairy Products in 1995 Obtained from 295 Million Dairy Cows

Overall milk production	528,616,000 tons
Milk deliveries to dairies	407,367,000 tons
Liquid milk including sour milk	107,703,000 tons
Cheese	14,908,000 tons
Butter	5,968,000 tons
Milk powders	5,192,000 tons
Condensed milk	3,126,000 tons

Source: Reprinted with permission from The World Dairy Situation 1995, *Bulletin of the International Dairy Federation*, Vol. 303, pp. 2–36, © 1995, International Dairy Federation.

Table 23-8 The Relative Importance of the Various Enzyme Sources for Proteolysis in Cheese

	Contribution to Proteolysis by						
			Internal Lactic Acid Bacteria		*Surface*		
Cheese Type	*Rennet*	*Plasmin*	*Mesophilic*	*Thermophilic*	*Coryneform*	*Mold*	*Internal Mold*
Butter cheese	+++	sl	++	–	–	–	–
Camembert	+++	±	++	–	–	+++	–
Munster	+++	±	++	–	+++	–	–
Gouda	+++	sl	+++	–	–	–	–
Cheddar	+++	sl	+++	–	–	–	–
Emmental	±	+/++	±	++	–	–	–
Gruyère	±	+/++	±	++	+	–	–
Provolone	+	+	–	++	–	–	–
Roquefort	++	±	++	–	–	–	+++
Gorgonzola	++	±	++	++	+	–	+++

Note: Importance: –, no; sl, slight; ±, some; +, considerable; ++, great; +++, very great.

Caucasus, koumiss in Central Asia, dahi in India, and leben in Egypt and Palestine. The acidification in the sour milk product (pH 4.0 to 4.5) brings about a precipitation of the casein in the form of fine flocs.

The definition of sour milk products is that they are obtained by the use of lactic acid bacteria (lactococci and lactobacilli) without removal of whey after the end of fermentation. Special products may contain additional microorganisms like yeasts and certain probiotic bacteria. The initial substrate is pasteurized or thermized, homogenized milk such as whole milk, partially defatted milk, skim milk, milk the fat content of which has been adjusted by the addition of cream, and cream. The amount of dry matter in the milk can be raised by evaporation or by the addition of dry skim milk powder or, in the case of yogurt, with edible casein. The sour milk products include (1) milk soured with mesophilic cultures of lactic acid bacteria, eg, sour milk, curdled milk, sour cream, buttermilk, (2) milk soured with thermophilic lactic acid bacteria, eg, yogurt, and (3) milk processed with special mesophilic or thermophilic cultures, which contain, in addition to typical lactic acid bacteria, other bacteria or yeasts, eg, acidophilus milk, kefir, or koumiss.

For the preparation of curdled milk,[69,112,113] the fat-adjusted and pasteurized milk is cooled to 22° or 30°C, depending on the process, and is inoculated with 2% to 5% or 0.1% to 1% of cultures, respectively. The main cultures that are used consist of *Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, and/or *Leuconostoc cremoris*. Souring (to about pH 4.6) and coagulation take place in the souring tank in about 16 hours. The sour milk is then cooled to 7° to 10°C and packed (Table 23-9). In another process, the milk is packed immediately after the addition of the culture, and it is soured and curdled in the package. Souring at low temperatures promotes the development of aroma.

Sour Cream

To prepare this, pasteurized cream is adjusted to a fat content of 10% or 20% to 25%, homogenized, and incubated at 18° to 20°C with 2% to 4% of a creamery culture. After about 9 hours, a pH of between 5.1 and 4.9 has been reached. After this, the product is cooled to 4°C, packed, and stored cold. Post-fermentation pasteurization is applied frequently.

Yogurt[108,113]

The sour milk product yogurt originated from the Balkans and the Middle East. Goats', ewes', and cows' milk are used for its manufacture. Solid yogurt is a product of milk-white color with a porcelain-like smooth surface. It possesses a creamy solid gel-like consistency, can be cut, and does not release whey. It has a fresh lactic acid smell and its taste is characteristic, pleasant, full, and slightly to intensely sour. Yogurt is manufactured from heat-treated milk of various fat grades or from cream, and also from milk the dry matter of which has been increased by evaporation or addition of milk powder or casein, using cultures of lactic acid bacteria that are specific for this purpose. As a product ready for consumption, it usually contains large numbers of living lactic acid bacteria ($>10^6$ to 10^9 ml^{-1}).

Table 23-9 Comparison of Microbiological and Technological Parameters of Sour Milk, Fresh Cheese, Feta, and Harzer Manufacture

	Sour Milk	*Kefir*	*Yogurt*	*Quark*	*Cottage Cheese*	*Feta*	*Harzer*
Milk	cow	cow	cow	cow	cow	sheep/goat	cow
Pasteurization	+	+	+	+	+	+ or –	+
Conditioning of milk	—	—	evaporation at 90°C	—	—	—	—
Starter cultures:							
Lactic acid bacteria	mesophilic	—	thermophilic	thermophilic (or mesophilic)	thermophilic (or mesophilic)	thermophilic and mesophilic	thermophilic (or mesophilic)
Specific cultures	—	kefir grain	—	—	—	—	*P. camemberti* or *Brevibacterium*
Coagulation of milk:							
Lactic fermentation	16 h at 23°C	10–20 h at 22°C	2.5–3 h at 42°–45°C	3–5 h at 38°–40°C (15–22 h at 20°–30°C)	as quark		as quark
Rennet	—	—	—	(–)	(+)	40–50 min at 32°C	(–)
Cutting of coagulum				at pH 4.8	at pH 4.7		as quark
Size				10–20 cm	1–2 cm	2–3 cm	
Stirring of curd				1–2 h (or 6–12 h)	slow ≈ 30 min		
Cooking (scalding)	—	—	—	—	≈ 60°C 1–3 h	—	—
Handling of curd	—	—	—	at pH 4.6 separation from whey	curd washed with cold germ-free water	drainage of curd in molds at 14°–15°C	milling of curd (32% dry matter)
Salting	—	—	—	mixing with spices, fruit, if desired	salt, spices, and cream added	after 12 h several dry saltings to 4% NaCl	addition of 3–4% NaCl, 0.5–1.1% $NaHCO_3$, $CaCO_3$
Secondary fermentation	—	—	—	—	—	cheese blocks stored until slime formation on surface	curd formed to cheeses 2–3 days stored at 20°–25°C
Ripening	—	—	—	—	—	packed in cans with 6–8% brine, 2 weeks at 15°C	smearing with *Brevibacterium* 1 week at 12°–18°C, 90–95% relative humidity
Ready for consumption	immediate	immediate	immediate	immediate	immediate	2 months at 4°–5°C	
Final product:							
Weight	variable	variable	variable	variable	variable	≈ 15 kg	50–100 g
Water content	as milk	as milk	slightly lower	70–75%	about 75%	≈ 55%	≈ 60%
pH-value	< 4.6	≈ 4.4	≈ 4	≈ 4.5	≈ 5	≈ 4.6	≈ 5–7

The specific culture used for the manufacture of yogurt consists of thermophilic lactic acid bacteria from the species *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which live together in symbiosis. In place of *L. bulgaricus*, it is possible occasionally to use *L. helveticus*. The yogurt organisms are found in milk curdled in hot countries but can otherwise be isolated from calf stomachs. The two species of bacteria activate one another. *S. thermophilus* rapidly lowers the redox potential and produces some formic acid, which promotes the growth of *L. bulgaricus*, and some carbon dioxide. *L. bulgaricus* is more strongly proteolytic. It produces some of the amino acids and peptides required by *S. thermophilus*. Thus, the two species of bacteria grow in a mixed culture more rapidly and produce more aroma components than either of the strains alone. In a traditional yogurt culture, the two species are present in a numerical ratio of about 1:1. This composition can be adjusted by the choice of incubation temperature. Since *S. thermophilus* has its temperature optimum at 40° to 45°C and *L. bulgaricus* at 45° to 50°C, cultivation temperatures above 45°C leads to the faster multiplication of *L. bulgaricus*.

In the manufacture of yogurt, *S. thermophilus* multiplies faster at the beginning of fermentation and starts the souring process (this explains the name starter culture!). At an acid concentration of about 0.6%, its activity declines, to die out at about 0.8% of lactic acid. *L. bulgaricus* then takes over the souring process to produce a maximum concentration of 1.5% to 2% lactic acid. A mild yogurt ready for consumption contains 0.8% to 0.9% lactic acid, and a sour yogurt 1.0% to 1.3%. The fermentation products are mainly lactic acid [D(–) and L(+)] and small amounts of volatile fatty acids, carbonyl compounds, and alcohols. The characteristic taste of yogurt is evoked by a bouquet of aroma compounds, the synthesis of which starts at a pH of 5, falls off in the pH range of 4.4 to 4.3, and diminishes greatly at about pH 4. Prolonged storage at low values leads to loss of the most important aroma components. The characteristic component of the yogurt aroma is acetaldehyde, others are acetone, ethanol, butan-2-one, diacetyl, and ethyl acetate. The total acid in yogurt is made up of 59% lactic acid, 28% citric acid, 5.3% acetic acid, 2.4% formic acid, 2.3% succinic acid, and small amounts of other acids.

Manufacture of Yogurt (Table 23-9). Because of the extreme susceptibility of the yogurt microflora to inhibitors and antibiotics, the milk must be completely free of such contamination. It is adjusted to the desired fat content and necessary dry matter content. After this it is heated to 60°C and, to improve the taste and also to increase the firmness of the gel, it is homogenized at a pressure of about 200 bar and is finally heat treated in the pasteurizer at 75° to 95°C for between 5 and 40 minutes. After cooling to the ripening temperature of 42° to 45°C, 1.5% to 3% of yogurt starter culture is added to the milk. The subsequent steps differ depending on whether solid yogurt or stirred yogurt is being produced.

Solid Yogurt (Table 23-9). A rapid decrease of the pH and the incipient coagulation of the milk leads to a steep rise in viscosity. Because a uniformly solid structure not releasing whey is required, after inoculation the milk must be filled into containers within 20 minutes. Incubation is carried out at 42° to 45°C for 2.5 to 3 hours. When a pH of 4.6 has been reached, the product must be cooled as rapidly as possible to temperatures below about 5°C. This substantially reduces the metabolic activities of the yogurt flora in order to avoid oversouring. Slight post-souring takes place even at these temperatures. From the sealing of the yogurt containers until cooling, shaking must be avoided at all costs in order to prevent the appearance of fissures in the gel that is forming, with an associated release of whey. Before filling of the starter culture–containing yogurt-milk into containers, flavoring components (preferentially pasteurized) of diverse types in the form of fruit, honey, jam, and others, can be added to the containers to produce a range of products.

Stirred Yogurt. In the manufacture of stirred yogurt, incubation is carried out in the ripening tank. Shortly before a pH of 4.7 has been reached, the tank is cooled so that the yogurt can be stirred at pH values of 4.7 to 4.6 at about 20°C. The substrate is then cooled in a heat exchanger to 5°C and is either filled directly or is first mixed with flavoring components and then filled into the selling containers. Because of the tendency to post-souring, storage must take place at 5°C or lower.

Kefir

This originated from the Caucasus and was obtained from cows', goats', or ewes' milk. Today, kefir is produced in large amounts, particularly in Russia, where in 1971 about 1,116,000 liters of whole-milk kefir was produced. In many countries, the popularity of kefir is only slightly less than that of yogurt. Kefir is a thick-flowing, creamy, slightly effervescent or foaming beverage of milk-white color. It contains 0.8% to 1% of lactic acid, 0.3% to 0.8% of ethanol, and carbon dioxide. The alcohol and carbon dioxide, together with small amounts of diacetyl, acetaldehyde, and acetone, contribute greatly to its characteristic refreshing taste. Today, kefir is manufactured from pasteurized milk or cream, the fat content or dry matter content of which has been adjusted by the use of cultures specific for this purpose.

The kefir cultures or kefir grains (Figure 23-5) are whitish-yellow clumps of pea to walnut size with cauliflower-like shape. These agglomerates, the structure of which consists of the sparingly water-soluble but highly swellable polysaccharide kefiran and acid-coagulated casein, contain a symbiotic microflora. In addition to lactose-fermenting yeasts[37] such as *Saccharomyces kefir* and *Candida kefir* (5% to 10% of the culture), it contains homofermentative and heterofermentative lactobacilli (eg, *Lactobacillus kefir*), mesophilic lactococci, flavor-forming *Leuconostoc,* and acetic acid bacteria (mainly *Acetobacter pasteurianus*). The composition of the mixed kefir culture is variable according to the process, the climate, or the milk used without, however, this giving rise to fundamental changes.

Manufacture of Kefir (Table 23-9). The prepared milk is heated in the heat exchanger to 70°C and is then homogenized and heat treated in the pasteurizer at 85°C for 30 minutes. Pasteurized milk is cooled to the fermentation temperature of 18° to 28°C, usually 22°C. In the ripening vessel 3% to 5% of kefir culture is added to the milk and, depending on the amount of inoculum, it is incubated for 10 to 20 hours. When a pH of 4.6 to 4.4 has been reached, the soft coagulum is stirred. The kefir grains are then separated off. These may, after being washed well with cold germ-free water, be added to a new production batch. The kefir is cooled to 7° to 12°C and is then filled either into glass bottles or plastic beakers and sealed. It can be sold directly for consumption or it can be left to post-ripen at 7°C for 24 to 48 hours, depending on the taste. When an airtight seal is opened, kefir tends to foam. If kefir is stored in bottles the closure of which permits carbon dioxide to escape, alcohol is also lost from the product. Kefir filled into plastic containers tends to cause the containers to swell and blow. This is a normal behavior for a living fermented product and does not mean that the product is spoiled.

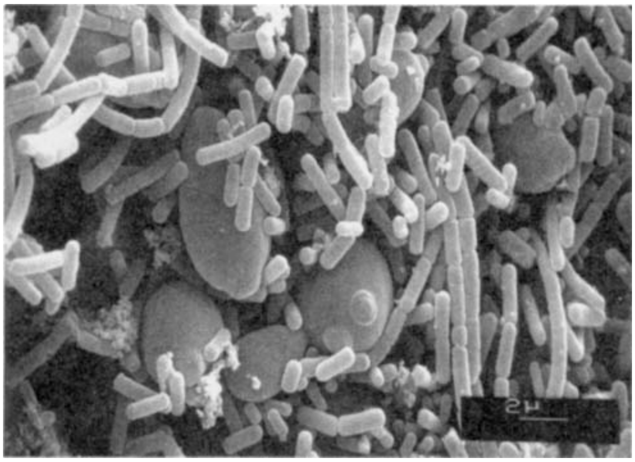

Figure 23-5 Scanning Electron Micrograph of the Surface of a Kefir Grain Showing Budding Yeast Cells, and Long and Short Rod-Shaped Lactic and Acetic Acid Bacteria. *Source:* Reprinted with permission from H. Neve, Analysis of Kefir Grain Starter Cultures by Scanning Electron Microscopy, *Milchwiss,* Vol. 47, pp. 275–278, © 1992, Volkswirtschaftlicher Verlag.

Koumiss

Koumiss, a homogeneous, slightly foaming, sour milk drink, comes from Central Asia. It was originally prepared from mares' milk (composition in Table 23-2). The product is recommended today, particularly in Russia, for dietetic treatment in the case of intestinal diseases because of its wholesomeness, ready digestibility, and vitamin content. Its taste is due to a simultaneous lactic acid and alcohol fermentation, and the taste-affecting metabolic products of the organisms of the koumiss culture. Koumiss contains 0.7% to 1.8% lactic acid, 0.5% to 2.5% ethanol, and carbon dioxide. In 1980 in Bashkir, 200,000 mares were available for the production of milk. In addition to this, modified cows' milk is being used in increasing amounts. Cows' and mares' milks have substantially different chemical compositions. In particular, the lower protein content in mares' milk and the ratio of casein to whey proteins of approximately 1:1 results in only partial coagulation of the milk, which is characteristic for koumiss. Consequently, the composition of skimmed cows' milk intended for the manufacture of koumiss is brought close to that of mares' milk by the addition of whey proteins and it is then fermented.

The cultures used are mixed cultures of yeasts (*Torula, Kluyveromyces, Candida,* and *Brettanomyces*), lactobacilli (*Lactobacillus delbrueckii* subsp. *bulgaricus, L. acidophilus*), and lactococci and streptococci *(Lactococcus lactis, S. thermophilus).* Yeasts and mixtures of bacteria are preincubated separately and are then added to the milk in ratios of 1:1–1:10. In principle, the manufacture is similar to that of kefir.

Presently, in many countries of the world, sour milk products containing typical inhabitants of the human gut (*Lactobacillus acidophilus, L. johnsonii, L. casei* subsp. *rhamnosus, Enterococcus faecium, Bifidobacterium bifidum,* and other bifidobacteria) are on sale (Section 23.6). Because these bacteria have physiological properties differing from those of traditional starter cultures, production of such fermented products needs specific fermentation conditions as exemplified for *Lactobacillus acidophilus.*

This bacterium has its temperature optimum at 35° to 38°C, the organisms of the cream-souring culture have theirs at 25° to 27°C, and those of yogurt at 40° to 48°C. It is therefore understandable that when *L. acidophilus* (or the closely related *L. johnsonii*) is added to normal starters and these mixed cultures are used under the conditions normal for the production of sour milk or yogurt, the resulting products only contain low numbers of *L. acidophilus.* Consequently, other ways have been found for the manufacture of *L. acidophilus*-containing milk products, eg, by fermentations at reduced temperatures (35° to 40°C). Alternatively, the different starter bacteria are fermented separately under optimum conditions. The final products are blended after fermentation.

Storage Life of Sour Milk Products[107]

The increasing popularity of sour milk products, the concentration and expansion of production units, and specialization of the factories for production of single types of product are leading to an increasing supply and more widespread and distant markets. This leads to a demand for an improvement in the shelf life of these products. The elimination of contaminants (eg, coliforms, yeasts, and molds) must be achieved by recontamination-free fermentation of the pasteurized substrate milk coupled with aseptic filling. Post-souring can be prevented only by post-fermentation pasteurization or sterilization of the fermented products. Although such sour milk products are on the market, sterilization of products, resulting in a very long shelf life, may not be desirable because these are essentially regarded as "fresh" foods, and would lose their desirable or organoleptic properties during long storage even under sterile conditions. In some countries, a "pasteurized or sterilized yogurt" must not carry the name yogurt, which is reserved for products containing living lactic acid bacteria. Post-fermentation pasteurized "yogurt" may in such countries be labeled as "sour milk product."

23.4.3 Cheese[27,46,101,136]

Cheeses are fresh products or products in various stages of ripening that have been manufactured from curdled milk. In contrast to sour milk products, the curdling of the milk is achieved either by acidification or by means of proteolytic enzymes such as calf rennet, pepsin, or microbial rennet. A further difference is that in cheese part of the aqueous phase of the milk is eliminated as whey during processing. The larger the amount of whey removed, the harder is the consistency of the cheese and the higher is its dry matter content. The degree of elimination of whey is determined essentially by the syneresis (separation of hydrophobic colloids) of the coagulum or curd (coagulated casein). The degree of syneresis, and therefore the release of whey during cheese manufacture, is increased by mechnical stirring and, in particular, by heating of the coagulum. The manufacturing process for a cheese can be subdivided into the following steps:

1. **Preripening of the cheese milk.** Raw milk or pasteurized milk is inoculated with a small amount (0.1%) of a culture of lactic acid bacteria and is incubated overnight at 12°C. This preripening step acts essentially as a process of conditioning the milk for the manufacture of cheese. The exact biochemical processes that occur are not yet known. Undoubtedly, an important factor is the resulting decreased redox potential of the milk. In the production of raw milk cheese, this step may lead to the multiplication of undesirable and pathogenic bacteria if present.[29]
2. **Renneting of the milk and curdling.** The preripened milk inoculated with starter culture is coagulated either by lactic fermentation alone (see *Fresh Cheeses* below) or by the addition of a rennet preparation.
3. **Cutting of the coagulum.** This process is necessary in order to induce the release of the whey and to allow formation of cheese loafs.
4. **Heating/cooking (scalding) of the cheese curd.** Heating has the aim of promoting syneresis (see above) and, consequently, the release of whey. The larger the amount of whey to be removed, the longer and the hotter the heating process. Temperatures above 45° to 50°C reduce the survival of enteric bacteria and yeasts. Thus, lowering of the so-called cooking (scalding) temperatures could possibly produce microbiological risks if raw milk is used for cheese making.
5. **Elimination of the whey and formation of the cheese mass.** In the case of soft and hard

cheeses, the whey is removed by mechanical means (eg, sieves or cheesecloth), in the case of fresh cheeses curd separators (centrifuges) are used for this purpose.

6. **Pressing the cheese into forms.** This process has the aim of eliminating more whey, after the final shaping of the cheese. During pressing, lactic acid fermentation proceeds further.
7. **Salting the cheese.** If the curd has not been dry salted before the formation of the cheese blocks, the shaped and molded cheeses are immersed for some time (a few hours to days) in a salt bath (brine is normally a concentrated or saturated solution of sodium chloride in water). Certain types of cheese can be dry salted (eg, Roquefort, cheddar).
8. **Ripening of the cheese.** Ripening aims to condition the final cheese product to produce the desired consistency, taste, and aroma. The microbiology and biochemistry of cheese ripening is not yet known in all its details. Important aspects are listed in Tables 23-9, 23-10, and Exhibit 23-2.[31]

Mair-Waldburg[88] lists hundreds of varieties of cheese. Below, the manufacture of some characteristic varieties of cheese are described in more detail.[45,88,111]

Fresh Cheeses

Fresh cheeses are natural, unripened products. They are manufactured from pasteurized milk of adjusted fat content by the use of starter cultures and rennet or rennet substitutes or with cultures alone by the precipitation of the casein and the separation of whey, and are marketed in the fresh state. Shelf life can be improved by heat treatment (pasteurization) after fermentation. Fresh cheese may be modified by incorporating flavoring additives in the cheese mass. The fresh cheeses include quark and granular fresh cheese (cottage cheese).

Quark (Table 23-9). This forms the main fresh cheese product in a large part of Europe. It is manufactured only from pasteurized skim milk, the fat content of which has been adjusted or, for the higher-fat grades, from milk with added cream. Quark is prepared with souring cultures and small amounts of rennet (sour rennet quark) or starter cultures alone (sour milk quark). In the low-temperature process at 20° to 30°C (time of souring 15 to 22 hours), mesophilic cultures, creamery cultures, or single-strain cultures of *Lactococcus lactis* subsp. *cremoris* are used. In the warm process at 38° to 40°C (souring time 3 to 5 hours), thermophilic lactic acid bacteria (*S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) are used.

Exhibit 23-2 Important Aspects of the Cheese Manufacturing Process with Respect to Ripening

Curd preparation
- Amount of rennet
- pH
- Curd particle size
- Cooking temperature
- Acidification rate

Fat content

Starter type
- Thermophilic/mesophilic
- O-/ L-/ LD-/ D-type[a]
- Code/strain

Processing conditions
- Propionic acid fermentation
- pH at whey separation
- Amount of curd washing water
- Heating and kneading of acidified curd
- Brining the cheese or salting the curd
- pH of the cheese
- Mold growth in/on the cheese
- Bacterial surface flora
- Size and shape of the cheese
- Ripening time and temperature
- Measure of moisture loss during ripening

[a] O = starters do not contain citrate-fermenting strains.
L = starters contain *Leuconostoc.*
D = starters contain "diacetylactis."
DL = starters contain *Leuconostoc* and "diacetylactis."

Source: Reprinted with permission from G. van den Berg and F.A. Exterkate, Technological Parameters Involved in Cheese Ripening, *International Dairy Journal,* Vol. 3, pp. 485–507, © 1993, Elsevier Applied Science.

The manufacture of quark involves the following steps:

1. **Preparation of milk.** Milk of adjusted fat content from the primary stock tank is heated to approximately the temperature of pasteurization, homogenized, and then pasteurized at 72°C for 15 seconds or at 85° to 95°C for a few seconds, cooled to 20° to 27°C or 38° to 42°C, according to the process being used, and pumped into ripening tanks with a capacity of 15,000 to 50,000 liters.
2. **Souring and curdling.** In the souring tank, 0.5% to 2% of the starter culture in the low-temperature process and 3% to 4% in the warm process is added, with, if required, the rennet preparation, and the mixture is thoroughly stirred for

about 2 minutes. Then the milk is left to rest until it has curdled. Small amounts of rennet are said to accelerate the coagulation through slight proteolysis and therefore also to have a favorable effect on the stability of the consistency of the casein gel.

3. **Cutting and stirring.** After 8 to 12 hours in the low-temperature process, or 1 to 3 hours in the warm process, when a pH of 4.8 has been reached, the coagulum is cut by an integrated cutting and stirring device in the souring tank into sections with an edge length of about 10 to 20 cm and is slowly stirred. In another 6 to 8, or 1 to 2 hours, with souring proceeding further, whey slowly separates out and the coagulum settles in the whey.
4. **Separation.** As soon as the pH has declined to 4.65 to 4.6, the stirring is stopped and the supernatant whey is run off. After this, the quark and the residual whey are removed from the tank. The quark can now be heat treated at 60°C to stop the activity of the rennet and to kill recontaminants and starter bacteria, ie, to prolong shelf life. Then, at a temperature of 30°C, the substrate passes through the quark separator for the elimination of more whey and for the adjustment of the desired dry matter content of the product. After this the quark is cooled to 4° to 8°C.
5. **Mixing.** The quark is processed to a uniform consistency in a kneading and mixing machine. Pasteurized cream, spices, or fruit preparations may be incorporated at the same time. The finished product is first stored in a silo and is then fed into packaging devices where filling is carried out with the greatest possible efforts to avoid recontamination. Quark pumps, piping, separators, and mixing devices of the unit must be so designed that the acid-coagulated casein micelles are not damaged by shear forces, which would lead to a continuing release of whey.[69] At refrigeration temperature, shelf life is about 3 to 4 weeks. Pasteurized and aseptically packed quark may keep for the same length of time at room temperature. However, because milk always contains a few heat-resistant ascospores of *Byssochlamys nivea*,[39] outgrowth of these spores will spoil quark under these conditions within 5 to 6 weeks of storage.

Cottage Cheese. Cottage cheese is a fresh cheese specialty. In the United States (US), it forms the main fresh cheese product. Cottage cheese is prepared from pasteurized skim milk with the addition of starter cultures, and rennet, by the acid coagulation of casein, and by special further heat treatment producing a granular product. To improve its taste, it is mixed with cream and common salt, and filled into containers.

The manufacture of cottage cheese involves the following steps (Table 23-9):

1. **Preparation of the milk.** Skim milk is homogenized and pasteurized (72° to 75°C for 15 seconds).
2. **Fermentation.** The starter cultures used are a mixture of mesophilic bacteria, mainly *Lactococcus lactis* subsp. *cremoris*, a small proportion of *L. lactis* subsp. *lactis*, and from 1% to a maximum of 3% of aroma-forming agents (*L. lactis* subsp. *lactis* biovar *diacetylactis* and/or *Leuconostoc cremoris*). Larger proportions of aroma-forming agents in the culture would affect the product adversely by the pronounced formation of gas. To facilitate curdling and the release of whey and to improve the granular texture by proteolysis, small amounts of rennet may also be added to the milk. The amount of starter culture and the temperature determine the time until the milk curdles.

   Milk, starter culture, and any rennet are mixed directly in the cheese vat and the mixture is left unstirred until it curdles. However, the milk may also be preripened by the starter culture until a pH of 5.7 is reached. It is then pumped into the cheese vat with the addition of the desired amount of rennet. The vats contain between 5,000 and 12,000 liters of milk. They are provided with cutting and stirring devices and also with heatable jackets. In the pretreatment of milk, up to the stage of the coagulation of the casein, any introduction of gas bubbles, whether by entrainment during pumping or stirring or through gas-forming microorganisms, must be avoided because the production of curds containing bubbles of gas makes processing difficult and causes reduction of yield.
3. **Cutting and stirring.** The solid curd with a pH of 4.7, corresponding to 0.4% to 0.5% of lactic acid, is cut into pieces with an edge length of 1 to 2 cm. Slow stirring prevents the pieces of curd from coalescing again and promotes the release of whey. Pronounced turbulence in the vat, which leads to the abrasion of cheese dust from the pieces of curd, has to be avoided.
4. **Scalding.** About 30 minutes after cutting, the contents of the vat are heated quite slowly to

temperatures of 42° to 60°C by passing hot water or steam into the jacket of the vat with simultaneous stirring and are left under these conditions for some time. This promotes the uniform release of whey from the grains of curd, so that a firm elastic curd arises with no formation of skin. This process lasts 1 to 3 hours.

5. **Washing.** After this, part of the whey is run off and, with stirring, the grains are washed with cold germ-free water and finally with ice water, which cools it to 8°C, and the water is run off.
6. **Mixing.** The curd is mixed with cream and, if desired, with the addition of salt. Spices or fruit preparations may also be added. After this, the granular fresh cheese is filled into packages. Composition of cottage cheese is water, 78.5%; protein, 12.3%; fat, 4.3%; lactose, 3.3%; and lactic acid, 0.4%. Stabilizers (eg, gums and starch) and the preservative sorbic acid may be added. Granular fresh cheese, especially when it contains flavoring additives, should be stored at 5°C or lower in order to retain its organoleptic quality. Shelf life is several weeks.

Sour Milk Cheese, Harzer Cheese

Sour milk cheeses, in contrast to fresh cheeses, are made from lactic acid–coagulated milk (sour curd) with additional ripening stages. The production of these cheeses is limited and is illustrated with reference to Harzer cheese (Table 23-9). Harzer cheese was originally manufactured from sour milk quark, but today it is also made from quark that has been obtained by a mixed rennet–lactic acid coagulation process (see above). The lactic acid fermentation can be carried out with mesophilic cultures (*Lactococcus lactis* subsp. *lactis* and *cremoris*) or with thermophilic cultures (*Lactobacillus helveticus, S. thermophilus,* etc). The dry matter content should be at least 32%. Quark from various productions and manufacturers is milled to walnut size and mixed with 3% to 4% of salt (NaCl) and 0.5% to 1.5% of ripening salts ($NaHCO_3$, $CaCO_3$). This is intended to neutralize part of the lactic acid in order to give a pH of 4.8 to 4.9. A further decomposition of the acids by yeasts (see section on surface ripened cheeses) is achieved by storage at room temperature for between several hours and one day. After fine comminution, the curd is shaped in special machines. The freshly formed cheeses are incubated on boards at 20° to 25°C in 95% atmospheric humidity for 2 to 3 days. During this process, the surface of the cheese is deacidified by film-forming yeasts. After the formation of a light yellow smooth skin, the cheeses are coated on all sides with a solution of 50 to 75 g of salt (NaCl) and 50 to 100 ml of a culture of *Brevibacterium linens* in a liter of water. After drying overnight, the cheeses are transferred to the ripening room where, at 12° to 18°C and a relative humidity of 90% to 95%, the typical red streak is formed. The cheeses can then be packaged and marketed.

Rennet Cheeses

The main varieties of cheese are made from rennet-coagulated milk. They are distinguished on the basis of their dry matter content as hard, semi-hard, and soft cheeses (Tables 23-10 and 23-11). Because there are many detailed recent reviews,[45,111] the present discussion concentrates on the basic description of some typical and important traditional cheese varieties that dominate modern cheese production and that demonstrate the microbiological, biochemical, and technological principles of cheese production, namely Emmental (Swiss) cheese, cheddar cheese, Romadour as an example of a surface-ripened brick cheese, Roquefort as an example of a blue mold–ripened cheese, Camembert as surface-ripened white mold cheese, mozzarella as a product heated after fermentation (pasta filata), and feta as a pickled cheese. These production outlines are the basis for understanding the resulting microbiological and hygienic problems described at the end of this chapter.

Emmental (Swiss) Cheese.[127] This cheese type is often regarded as the "King of all cheeses" because it represents the biochemically most sophisticated and hygienically as well as organoleptically most accepted product made from raw milk. The underlying biochemical reactions (lactic acid fermentation, CO_2 production leading to hole formation, and propionic acid fermentation) are given in Figures 23-6, 23-7, and 23-8. For the steps in the manufacture of Emmental cheese see the outline in Table 23-10.

Emmental cheese is produced mainly in Switzerland, Germany, Finland, France, and Austria from raw milk, but increasingly from pasteurized milk on a worldwide basis. There is a classical procedure of manufacture in the cheese vat in which 1,000 liters of milk give one large loaf of cheese. Currently, however, many large-scale manufacturers process up to 12,000 liters of milk in each cheese vat. The evening milk preripened overnight (12°C, temperature reached by the evening milk when cooled overnight with tap water) is mixed with the fresh morning milk. The added starter culture consists of 1% to 2% of a culture of thermophilic streptococci (*S. thermophilus*) and lactobacilli (eg, *Lactobacillus*

Table 23-10 Comparison of Microbiological and Technological Parameters in the Manufacture of Typical Rennet Cheeses

	Romadour	*Camembert*	*Mozzarella*	*Cheddar*	*Emmental*	*Roquefort*
1. Milk	cow	cow	buffalo	cow	cow	sheep
2. Pasteurization	past.[a]	past.	raw or past.	raw or past.	raw	raw
Conditioning of milk/ preripening	—	60 min at 30°–32°C	—	—	overnight 12°C	—
3. Starter culture						
Lactic acid bacteria	mesophilic	mesophilic (thermophilic)	mesophilic and thermophilic	mesophilic	thermophilic	mesophilic and thermophilic (artisanal!)
Specific cultures	*Brevibacterium*				*Propionibacterium* spp.	
4. Coagulation of milk by rennet	similar to Camembert	10–20 min at 30°–35°C	20 min at 35°C	≈30 min at 30°–32°C	30 min at 30°C	120–150 min at 30°C
5. Cutting of coagulum size	similar to Camembert	1.5–2 cm	1–1.5 cm	≈1 cm	1 cm	1–3 cm
Stirring/setting of curd		3–5 min setting	30 min setting 15 min stirring	10–20 min at 30°–32°C	45 min at 30°C	few times in several hours
6. Cooking (scalding)	similar to Camembert	none 5–10 min stirring	–whey drainage –matting of curd –scalding with hot water (75°–80°C)	30–35 min at 38–39°C 20 min stirring	30–40 min at 55°C 30–60 min stirring	none
7. Pressing of curd and specific handling	similar to Camembert	by own weight in molds several hours	–curd: 58°–60°C –cooling in water to 4°C	–whey drainage –matting of curd –cheddaring for 2–2.5 h	whey drainage with cloth, pressing overnight 1200 kg	curd filled into molds whey drainage during 4 days, transport to Roquefort
8. Salting	similar to Camembert	18–20% NaCl brine 30–120 min	12°–15°C 16–20% NaCl brine up to 12 hours	–milling of curd –2.5%–3% NaCl added –18–24 h pressing in molds	at pH 5.25 2–3 days at 15°C in brine, 2 weeks drying	dry salted by hand several times during 3 to 5 days at 9°–10°C
9. Secondary fermentation or specific handling	growth of *Brevibacterium* 2–3 weeks at 15°C	growth of mycelium at surface			6–8 weeks at 22°–24°C (propionic fermentation)	–surface brushed to remove salt-tolerant flora –piercing of cheeses

continues

Table 23-10 Continued

	Romadour	*Camembert*	*Mozzarella*	*Cheddar*	*Emmental*	*Roquefort*
10. Ripening	2–3 weeks at 10°–12°C	1–2 days at 16–18°C 2–3 weeks 14–16°C	packed in brine	8–10 days at 15°C 2–6 weeks at 10°C 1–5 months ≈7°C	at 10°–13°C	–cheese stored on edges, 18–23 days at 8°–18°C, 96% r.h. –cheese packed into tin foils for anaerobic ripening, 3 months at 7°–8°C, further storage at 1°C for 5–10 months
Ready for consumption	4–6 weeks at 6°–8°C	3–6 weeks	immediate	3–12 months	4–12 months	
11. Final product weight		80–120 g	0.1–1 kg	15–40 kg	60–130 kg	10–12 kg
Water content	55%	≈ 55%	—	≈ 34%	≈ <38%	≈ 50–55%
pH value	about 6 to >7	> 6 to >7	5.0–5.2	≈ 5.2	≈ 5.6	> 6
a_w-value	—	≈ 0.99	≈ 99 (brine)	≈ 0.96	≈ 0.95	> 0.96

[a] Pasteurized.

Table 23-11 Water and NaCl Content, pH Values and Water Activities of Cheeses Bought on the Swiss Market

Cheese	*Water %*	*NaCl % i.T.*	*pH Value*	*Water Activity*
Hard cheeses				
Bergkäse	23.5	3.10	5.59	0.886
Emmentaler	35.2	1.05	5.63	0.973
Greyerzer	36.3	2.26	5.25	0.957
Parmesan	28.9	3.46	5.25	0.892
Sbrinz	28.8	2.91	5.54	0.900
Semi-hard cheeses				
Appenzeller	40.6	3.08	6.27	0.972
Bel Paese	46.1	4.89	5.11	0.957
Cheddar	34.5	3.08	5.17	0.959
Edamer	45.1	5.90	5.34	0.947
Fontal	42.0	4.07	5.26	0.961
Gouda	39.6	3.84	5.11	0.953
St. Paulin	45.8	3.41	5.34	0.971
Tête de Moine	39.0	3.54	5.46	0.964
Tilsiter	38.5	3.30	5.65	0.961
Raclette	39.3	4.15	5.64	0.955
Belle de Champs	50.7	3.85	5.66	0.987
Brie suisse	48.7	3.66	5.55	0.974
Camembert suisse	50.4	5.42	7.39	0.990
Tomme vaudoise	52.1	3.42	6.52	0.987
Soft cheeses with red smear				
Limburger	54.9	6.54	6.77	0.982
Münster	46.2	2.32	5.29	0.970
Vacherin Mont d'Or	53.4	1.76	5.08	0.990
Frischkäse				
Cottage Cheese	80.6	4.54	4.88	0.984
Speisequark	70.5	0.78	4.68	0.990

Source: Adapted with permission from M. Rüegg and B. Blanc, Beziehungen zwischen Wasseraktivität, Wasser-Sorptionsvermögen und Zusammensetzung von Käse, *Milchwiss*, Vol. 42, pp. 194–201, © 1977, Volkswirtschaftlicher Verlag.

helveticus or *L. delbrueckii* subsp. *lactis*) in whey. In addition, a few drops of a culture of propionic acid bacteria must be added. The curdling time with rennet is about 30 minutes. The cutting of the curd (to 1 cm) and preliminary curd formation require 45 minutes. The mixture of curd and whey is heated with stirring (called cooking) to 53° to 55°C within 30 to 40 minutes and is stirred for another 30 to 60 minutes. At some stage here as the curd reaches a temperature of about 38° to 40°C, the starter bacteria begin to multiply. After cooking, the grains of curd are about 2 to 3 mm in size. After the settling of the curd, it is taken up with a cloth and placed into molds. First, it is compressed under a load of 500 kg for 20 minutes, and then under 1,000 kg for 60 minutes, and finally under 1,200 kg. During this procedure, it must be turned several times. After overnight pressing, the lactose should have been fermented completely to lactic acid (Figures 23-6 and 23-7). The pH of the cheese is then about 5.25. After this, the cheeses are placed in a salt bath (saturated NaCl) for 2 to 3 days at 15°C. The cheeses removed from the brine are dried in the salting room, which requires about 10 to 14 days. The cheeses are transferred into the warm room for 6 to 8 weeks. At temperatures of 22° to 24°C, the lactic acid formed is fermented by the propionic acid bacteria to carbon dioxide, acetic, and propionic acid, which gives rise to the characteristic formation of "eyes" (Figure 23-8) and to the development of the characteristic taste of the Swiss cheese. After the conclusion of gas formation, the cheeses are ripened in the storage cellar at 10° to 13°C. During this time, the surface of the cheeses must be washed regularly to remove the fungal coating that forms. The optimum time of ripening is 6 months and the maximum time of storage is a further 12 months at 15°C.

Late-blowing of hard cheeses is caused by spore-forming bacteria, eg, *Clostridium tyrobutyricum*, which ferment the lactic acid not to propionic acid but to butyric acid and hydrogen.[143] These bacteria cannot be avoided in the milk when the cows are fed with fermented fodder (silage). For this reason, in the German and Swiss production the feeding of silage is forbidden if the milk is to be supplied for the preparation of Emmental cheese. If legal, *C. tyrobutyricum* spores may be eliminated by hydrogen peroxide/catalase, addition of lysozyme to milk[60] or bactofugation of the cheese milk.[77] The use of nitrate, as in the case of Gouda manufacture in Holland and Germany,[142] is not acceptable for Emmental cheeses because it is not permitted, and also because it inhibits the propionic fermentation. Another fermentation failure is the so-called "Nachgärung" (late fermentation) in which, after the "eyes" have been formed, the production of carbon dioxide does not stop. This leads to fissures in the cheese and therefore to a lowering of its commercial value, although not always to an effect on the taste of the cheese. The microbiological reasons for this occurrence are so far unknown, but it is probably caused by contaminating bacteria such as enterococci and certain proteolytically active lactobacilli from the raw milk.

Cheddar Cheese. Cheddar cheese is produced mainly in the United Kingdom (UK), the US,

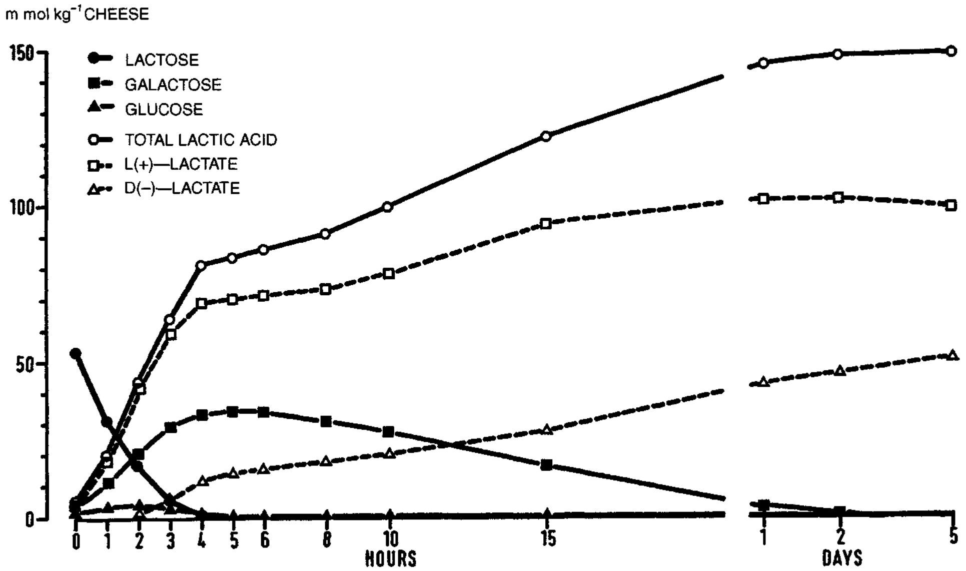

Figure 23-6 Carbohydrate Metabolism in a Young Emmental Cheese during the First Hours and Days of Manufacture. (See text and Table 23-10 for specific technological steps). *Streptococcus thermophilus* starts the fermentation by conversion of lactose into galactose and glucose, which is fermented to L(+) lactic acid within 4 hours. Galactose is excreted into the whey medium. *Lactobacillus delbrueckii* subsp. *lactis* (or *L. helveticus*) takes over the following fermentation converting galactose into D(−) lactic acid from 4 to 20 hours of fermentation, corresponding to the pressing stage. Courtesy of Swiss Federal Dairy Research Station, Liebefeld, Bern, Switzerland.

Canada, Australia, and New Zealand.[74] For steps in the manufacture see Table 23-10. The milk for its manufacture is pasteurized at 72.2°C for 15 seconds, cooled to 31°C, and is then treated with 1.5% of a single- or multiple-strain starter. Rennet (18 ml per 100 kg of milk) is stirred mechanically into the vat. The curdling time is from 30 to 35 minutes.

The curd is cut into cubes with a size about 1 cm^3. Five minutes after the end of this process, and within less than 30 minutes, the material is heated (scalded) to 38° to 39°C. The mixture is stirred at this temperature until the acidity in the whey reaches 0.15% of lactic acid (about 2.5 hours). The curd is allowed to settle and the whey is run off through filters. A rake-type stirrer is then drawn through the mass of curd several times during a period of 20 minutes in order to achieve the desired grain size. During this process, the acidity rises to about 0.23% to 0.24% of lactic acid. The mass of curd is now heaped up in order to achieve coalescence (cheddaring). After this procedure, pieces weighing 8 to 12 kg are cut out from this mass of curd and placed in layers above one another. During a period of storage of about 2 to 2.5 hours, the lactic acid content rises to 0.5% to 0.6% with a simultaneous drainage of whey. The cheddared blocks are then milled to form shreds of curd and are salted (about 3 kg of salt 100 kg^{-1} of cheese mass). Salted curd is transferred into cheese molds and pressed. After this, the cheeses are taken from the molds and coated with plastic foil or hot wax. In this form they are placed in the ripening cellar. They remain there at 7° or 10°C under a relative humidity of 55% for 1 to 12 months.

Romadour Cheese.[110] The pasteurized milk is treated with 0.2% to 0.5% of mesophilic starter. Then 15 to 20 ml of rennet solution (1:10,000) is added per 100 l of milk. The temperature at addition of the rennet is between 29° and 35°C, depending on the fat content. The whole curdling time amounts to 40 to 45 minutes. The curd is cut into 1.5- to 2-cm pieces. After 10 minutes, it is cut again and after 15 minutes some whey is taken off and the coagulum is agitated for 5 to 10 minutes in order to make it somewhat firmer. The coagulum is filled into perforated molds, and then kept for 24 hours in a salt bath (16% to 18% NaCl between 16° and 18°C). The cheese is then allowed to drain and dry. It is dried for another 2 to 3 days in the ripening cellar. Then it is smeared, ie, coated with a red-smear culture (*Brevibacterium linens*). Smearing must be repeated between three and eight times, depending on the size

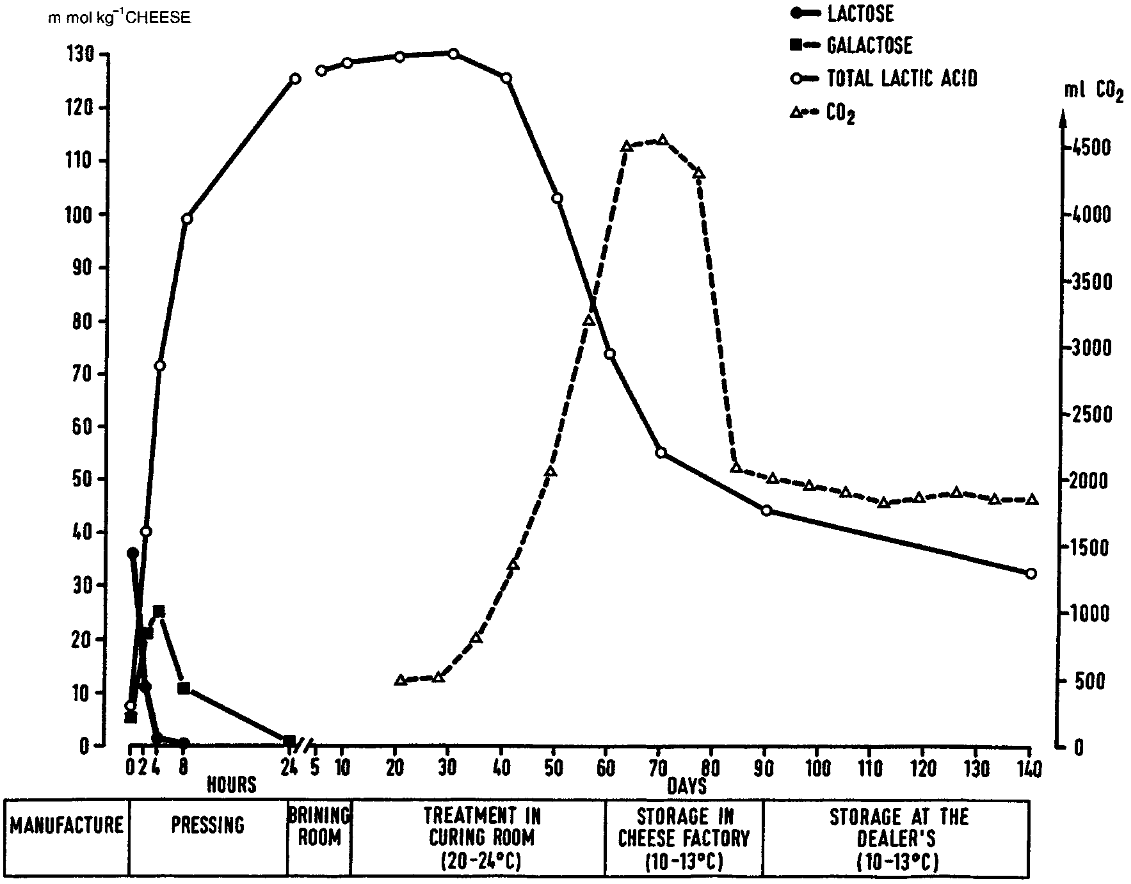

Figure 23-7 Production of Carbon Dioxide from Lactate during Ripening of Emmental Cheese. The underlying propionic acid fermentation is induced by transfer of the cheese loafs from a low temperature to the curing room at 20°–24°C. The CO_2 produced is responsible for the generation of the cheese eyes in Emmental cheese. Part of the early fermentation stage shown in Figure 23-6 is included. Courtesy of Swiss Federal Dairy Research Station, Liebefeld, Bern, Switzerland.

of the cheese, in order to obtain a thick surface growth of bacteria, particularly when the cellars are relatively dry and are at relatively high temperatures, otherwise growth of undesired molds takes place. Good ripening conditions are 14°C and a relative humidity of 90° to 95°C. After 3 to 4 weeks or, in the smaller cheeses after only 2 to 3 weeks, as soon as the red smear has developed well (Figure 23-9), the cheeses are packed in aluminum or paper foils. While in the classical cheese dairy the smearing of the surface was carried out by hand, today this task is performed mechanically. For steps in the manufacture see Table 23-10.

Camembert Cheese. This white-molded soft cheese variety is of particular interest for the cheese industry since ripening takes only 1 or 2 weeks, it can be produced in a variety of fat contents (about 20% to 65% fat in dry matter), and due to the relatively low dry matter content of soft cheese, the milk supply for the same amount of soft cheese is much lower than for a traditional hard cheese. In modern cheese technology, milk concentrated by ultrafiltration is commonly used for the manufacture of Camembert-type cheeses.[52]

Camembert is characterized by the development of the fungal mycelium at the surface and in the uppermost curd layers within the first weeks of cheese manufacture. The typical ripening progresses from the outside to the interior, induced by several proteases excreted from the mycelium. One end-product of this proteolysis is ammonia formed from deaminated amino acids. This leads to an increase in the pH value and to a characteristic smell (see *Microbiology of the Cheese Surface* in Section 23.7.2).

For steps in the manufacture see Table 23-10. Nowadays Camembert may be produced from raw milk in certain countries (eg, France), but normally

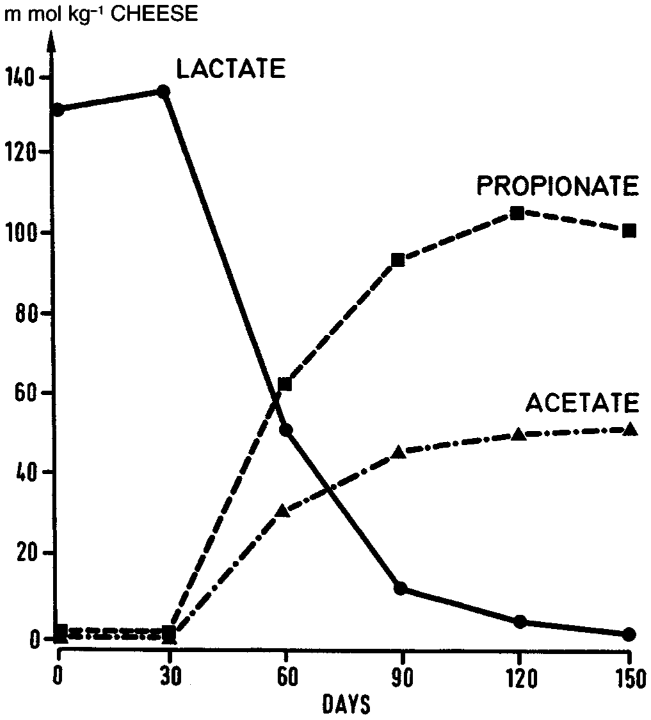

Figure 23-8 Course of the Propionic Acid Fermentation in an Emmental Cheese during Treatment in the Curing Room. Detail from Figure 23-7. Courtesy of Swiss Federal Dairy Research Station, Liebefeld, Bern, Switzerland.

from pasteurized milk. Other modern developments are the use of continuously working coagulators where the milk is curdled and the coagulum cut in an "endless" rubber trough, or the use of a thermophilic starter culture (*Streptococcus thermophilus*) instead of the traditional mesophilic cultures.[123]

Roquefort Cheese.[52] Traditional Roquefort cheese is produced from fresh raw ewes' milk in different parts of France. The average protein content of the milk is 6.5% and the average fat content is 8%. As a rule, artisanal cultures consisting of whey from the cheese production of the previous day, which contain a mixed microflora of lactic acid streptococci and lactic acid bacilli, are used as starter cultures. The optimum temperature for the growth of this culture is 35°C. *Penicillium roqueforti* is added to the cheese milk in the form of a suspension of spores. The renneting temperature is 30°C and the curdling time is 120 to 150 minutes with 25 ml of rennet (diluted 1:10,000) to 100 l of milk. The curd is cut into 1- to 3-cm cubes. During the next few hours, the curd is stirred a few times. Then it is allowed to settle and the whey is drawn off. The coagulum is filled into suitable stoneware or tinplate molds (diameter 20 cm, height 10 cm). The drainage of the whey from the molds lasts about 4 days, during which they are turned once a day. During this phase a slight heterofermentation of the lactic acid type takes place in the coagulum, which serves to create cavities for the development of the fungus. These still-soft cheeses are transported (from as far away as Corsica and the Pyrenees) to Roquefort. They are dry salted by hand with coarse sea salt in salting rooms (temperature 9° to 10°C) several times in the course of 3 to 5 days. The salt-tolerant microorganisms that develop are removed from the surface with brushes. Then the cheeses are manually or mechanically pierced with needles in order to allow access of oxygen to the mass of cheese and allow growth of the fungus. Ripening must by law take place exclusively in the natural cellars at Roquefort, which run for several kilometers through the mountain. The temperature in these cellars is constant at 7° to 10°C and the atmospheric humidity is between 90% and 100%. At first, the cheeses are stored in layers on their edges at 8° to 10°C and a relative humidity of 96% for 18 to 23 days. Yeasts and bacteria that develop on the surface of the cheeses are scraped off. After the end of this aerobic ripening, to induce the anaerobic phase, the cheeses are packed into tin foil

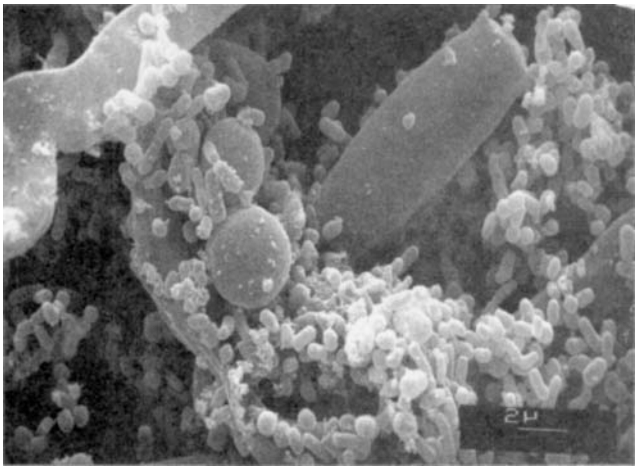

Figure 23-9 Scanning Electron Micrograph of the Surface of a Red-Smear Brick Cheese Showing Yeast Cells and Coryneform Bacteria. *Source:* Reprinted with permission from H. Neve and M. Teuber, Scanning Electron Microscopy of the Surface Microflora of Ripened Soft Cheeses, *Kieler Milchwirtschaftl. Forschungsberichte*, Vol. 41, pp. 3–13, © 1989, Bundesanstalt fur Milchforschung.

and stored at 7° to 8°C, again on their edges. Because of the lack of oxygen, the growth of the fungus slows down. Ripening, proteolysis, and lipolysis proceed further under the action of the proteolytic and lipolytic enzymes that have already been secreted into the cheese mass. This anaerobic phase lasts at least 3 months. A ripened cheese can be stored at 1°C for another 5 to 10 months. After the conclusion of ripening, the cheeses are taken from the tin foil and are packed in aluminum foil or plastic bags.

The high quality blue cheeses known in other European countries, such as Danish Blue, Bavaria Blue, and Bress Bleu, are made in a similar way from pasteurized cows' milk which, however, is otherwise treated as in the manner of Roquefort.

Mozzarella Cheese (see Table 23-10). This cheese belongs to the so-called pasta filata type which is a cheese traditionally made from raw buffalo milk.[8] Because the cheese curd is milled in hot water (80°–82°C) during the production process, a reduction of undesired microbes like enterobacteria takes place, although the time/temperature combination may not be equivalent to a true pasteurization. Modern production is mainly from pasteurized cows' milk. Remaining microbiological problems are recontamination during storage and handling in a whey-based brine.

Feta Cheese (Table 23-9). Traditional feta cheese is made from ewes' milk in Greece.[1] It has recently obtained legal protection within the European Union (EU). A similar cheese made from cows' milk must no longer be called feta. This cheese is made from a lactic acid/coagulant–produced cheese curd, which is stored in a whey/sodium chloride brine (16% to 23% NaCl) for several weeks to months. During this period, slow proteolysis and lipolysis take place yielding a typical flavor. The "ripening flora" involved is a secondary microflora containing salt-resistant species like *Enterococcus, Pediococcus,* and *Micrococcus.*

23.5 THE BACTERIOPHAGE PROBLEM IN DAIRY FERMENTATION[97,99]

23.5.1 The Extent of the Problem

Because sour milk and cheese are normally not made from sterilized milk and because most cheese fermentations are open systems (open vats, control of fermentation by direct and open inspection with hands, tools, etc), bacteriophages are a common threat. Even pasteurized milk may still contain virulent bacteriophages and residual lactic acid bacteria. The phages of the mesophilic lactococci and the thermophilic *S. thermophilus* have been investigated in detail. For the lactococci, at least 11 genetically distinct phage types have been described.[62] They may be lytic or temperate. Many starter culture strains of lactococci carry prophages that may be released during sour milk and cheese fermentations. In *S. thermophilus*, there seems to be only one genetic type of phage, which, however, reveals very distinct geographic variations and molecular specifications. The microbiology and genetics of these phages have been investigated thoroughly, leading to clear information on phage adsorption, injection, replication, and release. The host specificities have been found to vary substantially. Bacteriophage-resistant mechanisms have been characterized as being due to inhibition of adsorption, restriction/modification, or abortive infection. Many of these traits are coded for on plasmids and are therefore amenable to genetic engineering and transfers from cell to cell. In cheese factories, high bacteriophage levels in whey and curds are found under the following conditions, which may also lead to contamination with undesirable bacteria[43]:

1. Use of one centrifuge for whey and milk separation.
2. Use of whey cream for milk standardization, whey transfers by pumps, and casein fine savers in the area of raw milk silos, pasteurization equipment, and milk vacreators (gas removal devices).
3. Starter tanks located in the cheese vat room and entrance of bacteriophage to the tanks by air replacement during cooling or emptying.
4. Separate facilities for starter cans that have not been pressurized; the movement of air from whey handling areas to raw milk storage or cheese vat areas.
5. Whey stored in lines connecting vats (must be water- or air-flushed between vats).
6. Starter culture medium stored in lines connecting vats or lines from the pasteurizer and starter room. Lines should be kept at a minimum length.
7. Floor drains in the starter room (these should be eliminated) and drains in the cheese production room improperly flushed and sanitized during separate intervals of the day.
8. Improper cleaning of cheese vats between fills and lack of use of a sanitizer with at least 50 to 100 ppm available chlorine in the vat clean-in-place (CIP) system. Rinsing of vats between

refilling using whey rather than pure water contributes to a bacteriophage build-up and should be avoided.

9. Use of whey evaporator condensate or reverse osmosis permeate to wash tanks and cheesemaking equipment.
10. Use of long (excess of 1 hour) preripening times.
11. Use of non–phosphate-buffered media for bulk starter propagation.
12. Use of single-strain starter culture, if not properly protected.

In addition to the use of phage-resistant strains developed by genetic engineering or selected by challenge with bacteriophage cocktails, there are many generally accepted technological measures that reduce or avoid the bacteriophage problem. These include use of starters containing phage-unrelated or phage-insensitive strains, production of phage-free bulk starter, aseptic propagation systems, use of phage-inhibitory media, reduction of phage contamination of processing plants, starter culture rotations, air conditioning and free air flow, avoidance of aerosol generation, cleaning/chlorination of vats between refills, use of clean-in-place (CIP) systems, segregation of starter room and cheese process equipment, removal of deposits on bulk starter vessels, good factory design (suitable location of whey storage tanks, whey handling systems and wastewater treatment plants), regular use of an aerosol containing an agent such as chlorine to reduce contamination in the atmosphere, and other measures such as use of cultures for direct setting of the milk, inspection of jacket/agitators for pin holing, and early renneting.

Slow acidification due to bacteriophage attack of the starter culture may lead to good proliferation conditions for undesirable contaminant bacteria such as staphylococci, enterococci, listeria, enteric bacteria, and others. The use of strains resistant to specific phages may induce the selection of new, less virulent phages not encountered previously. The proper handling of the bacteriophage issue still remains a major challenge for proper dairy fermentations.

23.5.2 The *Lactococcus*-Specific Bacteriophages[56,132]

Bacteriophages are a common and constant threat to milk fermentation by lactococci, as most fermentations cannot be performed under sterile conditions, because heat sterilization damages the casein micelles, preventing proper coagulation and syneresis of the cheese curd. Even pasteurized milk may still contain phages and some residual lactic acid bacteria.

Hundreds of such phages have been isolated and described during the last five decades. However, only after progress in molecular biology could these phages be differentiated on the basis of DNA-DNA hybridization and protein profiles, for example. As a consequence, the International Subcommission on the Taxonomy of Bacteriophages of *Lactococcus* was able to agree on the establishment of defined bacteriophage species and type phages, which are available from an international reference laboratory.[62] The 11 defined species do not show detectable DNA homology, which is quite astonishing on the basis of the quite similar morphology especially of species commonly named "small isometric head" phages. Most of these species have been isolated as virulent or lytic phages. Only BK5-T is a temperate phage, and P335 reveals strong DNA homology with some temperate phages. Between 50% and 70% of the investigated bacterial host strains could be induced to release between one and three morphologically distinct prophage particles upon induction with mitomycin C or UV irradiation. Isolation of prophage-cured strains, however, is extremely difficult, because of immediate relysogenization of the cured derivatives in the induction assay. By electron microscopy, at least 19 morphologically distinguishable prophage particles have been detected. The taxonomic diversity of the lactococcal bacteriophages suggests, together with the observed diversity of lactococcal strains in mixed-strain starter cultures, a long and rapid evolution.

Biology of Host-Virus Interaction

Typically, milk to be fermented with *Lactococcus* is seeded with about 10^7 cfu ml^{-1}, which grow up to about 5×10^9 cfu ml^{-1} at the end of fermentation. Nine cell divisions will yield this increase. If only half of the world's cheese production is assumed to be manufactured with lactococci (ie, 7 million tons or 7×10^9 kg) this corresponds to 7×10^{10} liters of milk inoculated with about 10^{21} cfu proliferating to 10^{23} viable bacteria every year. Because cheese whey from a mixed-strain fermentation typically contains 10^6 to 10^8 phage particles ml^{-1} (even in the absence of a noticeable phage attack), up to at least 10^{21} phage particles are released during roughly 10^{22} cell divisions. This again provides ample opportunities for evolution of bacteria and phages.

Phage adsorption is usually not equally and densely packed over the whole bacterial cell surface, but to a few specific spots at the surface, possibly in the vicinity of the plane of cell division.[13] For that reason, the biochemical identification and isolation of phage receptor molecules has not yet been successful, although membrane lipoteichoic acid, cell-surface galactose- and rhamnose-containing polysaccharides, and murein complexes have been suggested to participate in phage adsorption. Latency periods after infection are around 90 minutes and burst sizes between 10 and 100 per infected cell.[99]

Most isolated virulent phages are very specific for a limited number of strains; a few, especially the prolate-headed phage species c2, may even cross the subspecies line between "lactis" and "cremoris." In addition, bacteria infected and lysed by such phages produce a highly active bacteriophage-coded lysin, which is able to lyse lactococci from the outside even if the strain is resistant to phage adsorption. For temperate phage particles released by mitomycin or UV induction from isolates from a mixed-strain starter culture, it is difficult to find sensitive host strains in that and in other starter cultures.This may reflect in part the situation that prophage-sensitive bacterial strains are immediately and constantly eliminated in a starter culture either by lysis or by lysogenization. *Lactococcus lactis* subsp. *cremoris* Wg2, a strain well known from investigations of the plasmid-coded cell wall–protease system, is exceptionally susceptible to many prophage particles (because of lack of a restriction/modification system?).

Distribution of Bacteriophage Resistance

When single isolates (23 each) of three undefined mixed starter cultures (A, B, C) were tested against 375 phage strains from our collection, only 34 phages, that is, fewer than 10%, showed lytic activities against one or more of the 69 bacterial strains. In all, 9 bacterial strains from culture A, 14 from culture B, and 2 from culture C were susceptible to one or more phages, the highest sensitivity being one bacterium from culture A, which was sensitive to eight different phages.[14] This observation suggests that phage-host interactions are rather specific. This is also evident from the specificity of phages isolated during the application cycles of the three starter cultures in a cheese factory. The phages appearing during cycle A were mainly specific for bacterial strains from culture A, and likewise for the cycles B and C. The distribution of strains with varying degrees of phage resistance in four different, undefined mixed-strain starter cultures is remarkable. A significant individual resistance level is evident. From a total of seven undefined mixed-strain starters, 30 permanently and highly phage-resistant strains were obtained by challenging with a phage cocktail containing all phages ever isolated in the author's laboratory.[93] This proves the high potential of lactococci to develop phage-resistant mechanisms, which actually allowed the successful use of such bacteria in spontaneous milk fermentations during the hundreds of years before the invention of starter cultures 100 years ago by Weigmann and others.

If the spatial distribution of phages in dairy plants is investigated, high numbers are found in whey and in the product (if it is not pasteurized). After enrichment, phages are detected in raw and pasteurized milk, on the surface of equipment, the clothes and skin of the cheese makers, and in the air, especially in the neighborhood of areas where whey-containing aerosols are produced, for example, close to whey separators. Cold disinfection of phage-contaminated surfaces and material is most effective with hypochlorite and peracetic acid.[76]

Mechanisms of Bacteriophage Resistance

Restriction of bacteriophages can be demonstrated commonly in lactococci, eg, with host-virus systems isolated from cheese factories. The observed efficiencies of restriction in plaque numbers are between 10^3 and 10^7. In addition, inhibition of phage adsorption has been reported quite regularly. However, because phage adsorption to lactococcal cells is difficult to determine and to distinguish from unspecific binding, due to the "hot-spot" mechanism, adsorption studies can only be trusted if controlled by electron microscopy. The most interesting phenomenon is the quite common effect of "abortive infection." This has been demonstrated unequivocally by the proof of phage adsorption and DNA-injection with phages carrying a genomic DNA labeled with the fluorescent dye DAPI.[49] In some systems, phage replication is inhibited as late as in the translation of phage messenger RNA into phage protein.

Plasmids and Bacteriophage Resistance

Plasmids are common in lactococci as discussed above. In ecological terms, lactococci are able to exchange genes necessary for better survival in milk. Phage-resistant plasmids of lactococci have been characterized and some of the genes coding for phage resistance have been cloned. In addition, phage-resistant plasmids have been transferred by conjugation to phage-sensitive starter culture strains. In the dairy industry in the US, use of strains showing resistance to phage species c2 and 936 has led to the enrichment of phages from the P335 species in

American dairies. These newly isolated phages (known in Europe for some time) had high numbers of restriction sites in their genome in contrast to the traditional c2 and 936 phage species. This observation is suggested to result from a selection during evolution for phage strains with no or few restriction sites, which are therefore able to escape the restriction/modification systems of lactococci.[92]

Molecular Biology of Lactococcal Bacteriophages

The study of the molecular biology of the bacteriophages of lactococci is in a rapidly developing stage. All phages hitherto isolated contain double-stranded DNA in a closed circular, linear, or permutated form. In terms of DNA sequences, we know the nucleotide sequences of a gene coding for a presumptive structural protein and of the structural gene for lysin in prolate-headed lactococcal bacteriophages of the c2 species. Several more or less strong promotors have been isolated from lactococcal phages with the use of promotor probe vectors. Some work has been done regarding the integration of temperate phages into the lactococcal chromosome. Also, the characterization of restriction/modification systems involved in phage resistance is in an early phase. An interesting approach is the use of antisense mRNA to induce bacteriophage resistance in lactococci.[71] Some genomes of temperate and lytic phage have recently been sequenced completely, allowing further insight into replication and integration of these phages.

Stability of Bacteriophage Resistance in Lactococci

The fact that many resistance phenomena are coded for on plasmids suggests that plasmid loss could lead to loss of phage resistance, which is indeed the case. In everyday cheese making, the loss of phage resistance of undefined mixed-strain cultures was unequivocally shown by the experience of Stadhouders and Leenders[126] in the Netherlands. As soon as the cultures were propagated in the laboratory in sterile skim milk in the absence of phages, the mixed-strain cultures were converted into a culture with just a few dominating strains. The previously high bacteriophage resistance, known from cheese fermentations, was completely lost within a short time. This observation emphasizes the need for a constant challenge to such cultures under manufacturing conditions in order to keep them in an optimal state.

23.6 FERMENTED DAIRY PRODUCTS CONTAINING "PROBIOTIC" MICROORGANISMS

The health significance of living microorganisms in fermented dairy products is most controversial at this time. It is discussed with passion by the scientific community, but also within the International Dairy Federation and the corresponding trade associations within the EU.[59,133]

The background is the fact that the traditional, spontaneously fermented dairy products contained living microorganisms that proliferated during fermentation. Induced by the concept of Metchnikov at the beginning of this century, people started to believe that living lactic acid–producing bacteria consumed with fermented dairy products (yogurt, kefir, sour milk) would have a beneficial effect on the intestinal microflora of man and animals. The mechanisms of action suggested were twofold: (1) the ingested microorganisms attach to the intestinal mucosa thereby displacing harmful bacteria, and (2) the ingested microorganisms multiply and produce inhibitors, which induce a decrease of harmful microorganisms or inhibit their multiplication and increase. There are a number of obstacles to this general belief. First, the lactic acid bacteria used for dairy fermentations are not members of the normal intestinal microflora. They are best adapted to their substrate, milk; they are not adapted to the substrates and environment present in different segments of the intestine. The intestinal sites are already populated by bacteria best adapted to them (eg, up to 10^{12} g^{-1} feces). It is almost impossible to invade such a crowded biotope successfully.[46] Components of the microflora of fermented dairy products have been shown to appear transiently at low numbers (10^3 to 10^5) in the feces as long as the fermented product is consumed regularly.[7,89] Experiments with enteropathogenic *Escherichia coli* strains given to volunteers together with or without fermented products containing *Enterococcus faecium* SF68 (Table 23-13) clearly showed no protection against enteropathogenic *E. coli* by the fermented products.

The obvious inability of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactococcus lactis* to establish themselves in the intestinal microflora of man and animal, led the supporters of Metchnikov's ideas to incorporate probiotic members of the intestinal microflora such as bifidobacteria, *Lactobacillus acidophilus*, *Enterococcus faecium*, and some other lactobacilli into fermented dairy products. The problem arose that these

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bacterial species are not well adapted to milk as substrate. This can be overcome by adding separately cultivated bifidobacteria and *Lactobacillus acidophilus* in high numbers to the milk before fermentation into sour milk, yogurt, and cheese.

The term *probiotic* was originally used to denote substances secreted by a microorganism that stimulate the growth of another, in contrast with the traditional definition of an antibiotic. Finally, Fuller[47] redefined probiotic as "A live microbial feed supplement which beneficially affects the host (animal) by improving its intestinal microbial balance." This definition has the following weak points, which still contribute to the scientific and public confusion regarding probiotics: (1) The beneficial effect can be interpreted so broadly that almost everything fits into it, (2) improving of a balance is not possible in a logic sense; therefore, some authors use the term *stabilization,* and (3) the intestinal microbial balance is not known for any individual human or animal being. It may be the situation when the individual feels healthy regarding its digestive function.

This lack of a clear definition has led to the situation in which lists of beneficial functions derived from the properties of probiotic microorganisms have been produced. The main contrast with traditional starter cultures is the fact that probiotic cultures have no function in the fermentation of the product but are claimed to have a function in the body of the consuming macroorganism. Table 23-12 lists the desirable properties of probiotic bacteria.[75]

The following probiotic microorganisms are used in some products that are on the market:

1. Several *Bifidobacterium* species (*B. longum, B. infantis, B. bifidum, B. breve, B. animalis, B. lactis*)
2. Several *Lactobacillus* species (*L. acidophilus, L. casei, L. delbrueckii* subsp. *bulgaricus, L. plantarum, L. reuteri, L. rhamnosus*)
3. *Enterococcus faecium*
4. *Propionibacterium freudenreichii*
5. *Saccharomyces boulardii*

Some of these microorganisms, if consumed with fermented dairy products, were found transiently in the intestinal microflora of human volunteers (up to 10^3 to 10^5 g^{-1} feces), but normally not exceeding the number of the naturally occurring related bacteria in the autochthonous flora of the consuming individual.

Recent research has concerned the prevention and treatment of diarrhea and urinary and vaginal infection. Results are controversial and inconclusive (Table 23-13).[34] There seems to be some protective effect in the treatment of diarrhea; it is, however, not possible to predict whether a specific person will benefit from the treatment.

The following additional claims are made for the beneficial effects of fermented dairy products generally, and bifidus and acidophilus products in particular on the consumer: (1) cultured products with living probiotic bacteria enhance the growth of young animals, and may protect them against dysenteries, (2) lactose digestion may be enhanced, which may be important for lactose-intolerant persons, (3) mineral (calcium) absorption might be increased, (4) the cholesterol level of blood may be reduced, (5) the ingested microorganisms colonize the gut, lower intes-

Table 23-12 Desirable Properties of Probiotic Bacteria

Health and Clinical Properties	*Stability and Technical Properties*
Human origin	Ability to maintain verified viability
Resistance to acid and bile	Maintenance of good flavor and aroma profile after fermentation
Adherence to human intestinal cells	Maintenance of mild acidity throughout storage; good acidity profile
Colonization of the human gut	Maintenance of colonizing properties throughout processing and storage
Production of antimicrobial substances	Development of good storage stability in fermented products
Antagonism against cariogenic and pathogenic bacteria	Stability after freeze drying and other drying methods
Safe for human consumption	Accurate identification of strains
Clinically validated health effects	Dose-response data for required effects

Source: Reprinted with permission from Y.K. Lee and S. Salminen, The Coming of Age of Probiotics, Review, *Trends in Food Science Technology,* Vol. 6, pp. 241–244, © 1995, Elsevier Applied Science.

Table 23-13 Use of Biotherapeutic Agents in Double-Blind Placebo-Controlled Trials in Humans

Biotherapeutic Agent	*Indication*	*No. of Subjects*	*Duration of Treatment (days)*	*Frequency of Disease*	
				In Treated	*In Controls*
Prevention of diarrhea					
Saccharomyces boulardii	AAD	388	7	4.5%[a]	17%
S. boulardii	AAD	180	28	9.5%[a]	21.8%
S. boulardii	AAD	193	28	7.2%[a]	14.6%
Lactobacillus acidophilus and *Lactobacillus bulgaricus*	AAD	79	5	0%[a]	14.0%
L. acidophilus and L. bulgaricus	AAD	38	10	66%	69%
L. casei GG	AAD	16	7	2 days[a,b]	8 days[b]
Bifidobacterium longum	AAD	10	3	10%[a]	60%
B. longum and L. acidophilus	AAD	20	21	20%[a]	70%
Enterococcus faecium SF68	AAD	45	7	8.7%	27.2%
B. bifidum and Streptococcus thermophilus	Infantile diarrhea	55	NG	7%[a]	31 %
L. casei GG	Traveler's diarrhea	756	7–14	41.0%	46.5%
S. boulardii	Traveler's diarrhea	1016	VAR	28.7%[a]	39.1%
Treatment of diarrhea					
S. boulardii	*Clostridium difficile* disease	124	28	26%[a]	45%
S. boulardii	HIV-related diarrhea	35	7	44%[a]	94%
S. boulardii	Acute diarrhea				
	Infant	130		15%[a]	60%
	Adult	98	4	4.9%	23.9%
E. faecium SF68	Adult acute	78	7	0.6%[a]	8.7%
E. faecium SF68	Adult acute	114	3	3.3 days[b]	3.3 days[b]
	Vibrio cholerae				
	Enterotoxigenic	41	3	2 days[b]	2 days
	Escherichia coli				
	Unknown etiology	28	3	NG	NG

Note: AAD indicates antibiotic-associated diarrhea, NG = not given, VAR = variable, HIV = human immunodeficiency virus.

[a] P > 05, compared with controls.

[b] Mean diarrheal days.

Source: Adapted with permission form G.W. Elmer, M. Surawics, and L.V. MacFarland, Biotherapeutic Agents, A Neglected Modality for the Treatment and Prevention of Selected Intestinal and Vaginal Infections, *JAMA*, Vol. 275, pp. 870–876, © 1996, American Medical Association.

tinal pH, adhere to the intestinal walls thereby preventing colonization by pathogens, compete for nutrients with pathogenic bacteria, produce antibacterial substances, and neutralize toxins, (6) cultured products have antitumor and antimutagenic properties, (7) cultured products interact with the immune system, and (8) relieve constipation, reduce transit time, and increase stool frequency.

It must be stated clearly that many of these claims could not be verified when the experiments were repeated by other laboratories, with other animals or persons, and with different products. The only generally accepted result seems to be the observation that lactose-intolerant persons are able to digest lactose properly if it is consumed in the form of yogurt. Sometimes this effect was only observed if living bacteria were present; sometimes the same effect was also seen with pasteurized yogurt.

Although a 400-page book was published on this subject[47] careful reading reveals that the book mainly describes problems and not solutions. In my opinion, we do not know enough about the intestinal microflora to be sure that consumption of fermented dairy products containing living bacteria does improve the microbial balance of our gut, whatever that means. Whereas we have a great deal of information regarding the microbiological and biochemical composition of fermented dairy products, most of the claims regarding "probiotic" action inside our body still remain fiction and wishful thinking.

That the administration of different *Lactobacillus* strains may indeed lead to *in vivo* colonization of the human intestinal mucosa was recently shown in a very controlled study where the bacteria were given in the form of fermented oatmeal soup. This study investigated the microflora of the intestine by taking biopsy samples from the upper jejunum and the rectum. Of the 19 tested bacterial strains, only 5 strains could be reisolated from the mucosa samples. The administered *Lactobacillus acidophilus* strains were not detected.[64] Only very few of the dairy strains have been tested with such an elaborate method, although they are added to many products. Further research is needed to assess the claims for probiotics.

Until this has been achieved, the summary statements of the very careful and critical review of Marteau and Rambaud[89] are still a valid description of the current situation: "There is in 1993 no proven medical indication of lactic acid bacteria for therapy or immunomodulation in man."

There are recent claims that specific "probiotic" bacteria induce a nonspecific stimulation of the human immune system such as increased IgA excretion after immunization with a *Salmonella* vaccine or increased phagocytosis of *E. coli* by certain phagocytes circulating in the blood. However, a control with ordinary yogurt has not been done.[83]

An exciting development is the use of lactic acid bacteria (ordinary lactococci and lactobacilli) as vaccine delivery systems. The first successful experiments in animals prove that such bacteria, live or dead, interact with the intestinal immune system, eg, protecting mice against tetanus toxin.[144]

These observations must be taken as an indication that possibly many bacteria, if not all, that are consumed with food could interact with the GALT (gastrointestinal associated lymphoid tissue). Therefore great care must be taken when applying new "probiotic" bacteria as a high level food supplement.[90] In summary, the following points must be observed if the "probiotic" concept is to be validated:

1. We need a clear, logical new definition.
2. We need a clean and thorough taxonomy of all the 400 to 600 different species suggested to grow in the human intestine, including the not-yet-culturable species.
3. We need to know the qualitative and quantitative distribution of microorganisms in the longitudinal (duodenum, small intestine, colon) and cross axis (mucosa, lumen) of the intestine.
4. The metabolism of food components *in situ* must be studied in the different sections of the intestine defined in point 3 above.
5. We are only at the beginning of understanding the immune system and its function in the intestine. We do not know much about the tolerance of the human body against its normal microflora. We know even less what happens if we introduce new "probiotic" bacteria. For example, the possibility of an anaphylactic event in probiotic-induced individuals has not been investigated.
6. We know too little about the influence of a normal diet and all its components on the intestinal microflora. The possibilities must be investigated that the changes attributed to application of probiotic bacteria might be obtained by stimulating specifically, for example, bifidobacteria, lactobacilli, or others, with specific food ingredients ("prebiotics").
7. We need more controlled and independent clinical research in addition to the basic microbiological problems still at hand.

23.7 THE MICROBIOLOGICAL SAFETY OF FERMENTED DAIRY PRODUCTS

23.7.1 Safety of Sour Milk Products

It is now common worldwide to produce yogurt, sour milk, sour cream, and fresh cheese from pasteurized milk. Because milk that has been properly pasteurized (see Codex Alimentarius) poses no health risks for the consumers, fermented milk products have the same safety level if post-pasteurization recontamination is prevented. Because of the lowered pH of fermented milk, the main risk is the growth of contaminating yeasts and molds. One contaminating yeast cell in a 250 g fresh cheese pack can multiply to 10^4–10^6 cfu g^{-1} within a storage period of 15 to 20 days at 5° to 10°C.[35] However, modern aseptic fermentation and packaging procedures are available to avoid contamination by yeasts and molds. Yeast spoilage is recognized by the consumer because of its "yeasty" taste and smell but it poses no health hazard. The development of visible mold growth on the surface of fermented milk products leads to rejection of the product. There is a need that fruit, nuts, and other ingredients added to sour milk, yogurt, or fresh cheese are themselves microbiologically safe to avoid hazards like the contamination with *Clostridium botulinum* toxin in a recent incident in Great Britain (Chapter 41).

23.7.2 Safety of Cheese

Outbreaks of Foodborne Illness Associated with Cheese

Table 23-14 lists the main outbreaks of foodborne illnesses associated with cheese that have been

Table 23-14 Reported Outbreaks of Foodborne Disease Associated with Cheeses Since 1980

Outbreak	*Pathogen*	*Number of Cases*	*Number of Deaths*	*Food*	*Reference*
1980, England and Wales	*Salmonella sp.*	6	NR	"untreated" goats' milk cheese imported from Turkey[a]	122
1980, US	*S. heidelberg*	>339	NR	cheddar cheese[b]	33, 44
1981, Italy	*S. typhimurium*	>100	0	mozzarella cheese[b]	53, 122
1982, Canada	*S. muenster*	NR	NR	cheddar cheese[c]	33, 146
1983, Finland	*Salmonella* sp.	35	NR	"farmhouse" cheese[a]	122
1984, Canada	*S. typhimurium*	>2,700	1	cheddar cheese[d,e]	21–24
1985, Switzerland	*S. typhimurium*	>40	0	Vacherin Mont d'Or cheese[b,c]	116
1989, England	*S. dublin*	42	0	Irish soft cheese[c]	87
1989, US	*S. javiana* and *S. oranienberg*	164[f]	0	mozzarella cheese[b]	33, 55
1990, France	*S. paratyphi B*	277	NR	"possibly related to goats' milk cheese"[a]	28, 51
1993, France	*S. paratyphi B*	273	1	goats'milk cheese[c]	28
1995, Switzerland and France	*S. dublin*	NRS, 25 in France	NRS, 5 in France	cheese from Doubs region of France[c]	139
1996, England	*S. gold-coast*	>84	0	cheddar-type cheese[e]	5
1982, Scandinavia	*Shigella sonnei*	>50	0	French Brie/ Camembert cheese[a]	53, 122
1983, Netherlands, Denmark, Sweden, US	enterotoxigenic *Escherichia coli*	>3,000	NR	Brie cheese[a]	85, 100
1992–3, France	verocytotoxin-forming *E. coli*	NR, 4 cases of HUS[g]	1	fromage frais[c]	2
1994, Scotland	verocytotoxin-forming *E. coli 0157*	>20[h]	0	local, farm-produced cheese[c]	3
1983, US	*Brucella* sp.	>30	NR	goats' milk cheese[c]	105, 122
1983–4, Greece	*Brucella* sp.	23	NR	"home-made, unripened cheese"[a]	122
1992, US	*B. melitensis*	11	0	goats' milk cheese imported from Mexico[c]	17
1995, Malta	*B. melitensis*	135	1	soft cheese made from sheep and goats' milk[c]	4
1983–7, Switzerland	*Listeria monocytogenes*	>122	34	Vacherin Mont d'Or cheese[b,c]	10
1985, US	*L. monocytogenes*	>142	48	Mexican-style cheese[d]	82, 115
1995, France	*L. monocytogenes*	20	4	Brie de Meaux cheese[c]	50
1983, France	*Staphylococcus aureus* enterotoxin	20	0	sheep's milk cheese[c]	122

continues

Table 23-14 Continued

Outbreak	*Pathogen*	*Number of Cases*	*Number of Deaths*	*Food*	*Reference*
1983, England and Wales	*S. aureus* enterotoxin	2	0	cheese[a]	122
1984–5, Scotland	*S. aureus* enterotoxin	>13	0	sheep milk cheese[c]	12
1983, US	*Streptococcus zooepidemicus*	16	2	Quesno Blanco[c] (homemade)	42, 53
1988–9, England	unknown cause	155	0	Stilton cheese[c]	86
1996, Italy	*Clostridium botulinum,* toxin type A	8	1	Mascarpone cheese[e]	6

Note: NRS = number in Switzerland not reported, NR = not reported.

[a] Not specifically stated whether the milk used for cheese production was pasteurized.

[b] Post-pasteurization contamination likely.

[c] These products are reported to have been preprepared using unpasteurized milk.

[d] Pasteurized milk was used, but there was evidence that unpasteurized milk was also included.

[e] Associated with faults in the pasteurization process.

[f] "It is estimated that 1500 to 15,000 outbreak-associated cases of salmonellosis may have occurred in Minnesota."[33]

[g] Four reported cases of hemolytic uremic syndrome (HUS).

[h] One case of hemolytic uremic syndrome.

documented in the literature for the period starting in 1980. A discussion of the safety of cheese was published recently.[58] Although cheese consumption amounts to at least 15 million tons of cheese per year in the world,[61] the reported rate of cheeseborne illness must be regarded as relatively low. On the other side, the recorded incidences include pathogenic bacteria such as *Clostridium botulinum, Listeria monocytogenes, Salmonella,* and *Brucella* species. As a consequence, cheese producers must aim at a further and constant watch of the hygienic conditions in cheese manufacture.

Microbiological Risks

Soft and semi-hard cheeses with a high water activity (Table 23-11), especially when made from raw milk, need extra attention including investigation of the presence of antibiotic-resistant bacteria. Post-pasteurization contamination is the main risk of cheeses made from properly heat-treated milk. It must be recognized, however, that cheeses made from raw milk present the main sources of cheeseborne outbreaks. One reason is surely the fact that soft cheeses do not provide sufficient hurdles (pH value, water activity) to prevent the growth of pathogenic bacteria once they have contaminated or recontaminated the cheese milk and the cheese itself. In hard and semi-hard cheeses, there may be an inhomogenous structure with no regular distribution of water content or lactic/propionic concentration allowing the growth of undesired microorganisms in such niches. The cheese rind as a natural cover (see below) is another microbiological problem.

Obviously, the microbiological safety of cheese cannot be properly controlled if raw milk or thermized milk is used rather than pasteurized milk. Other methods of reducing the microbial load of raw milk like bactofugation, microfiltration, or use of additives (H_2O_2 and catalase, or carbon dioxide), do not achieve the same effect as pasteurization by heat. It is important to prevent post-pasteurization contamination, which may result in survival and growth of pathogens during manufacture and ripening of cheese. The risks of pathogens in cheese have been assessed into high, medium, and low risk categories.[65–67]

High Risk

- *Salmonella* species: Involved in several cheese outbreaks; widespread in environment, low infectious dose, some heat-resistant species. Survives in cheese.
- *Listeria monocytogenes*: Involved in several outbreaks associated with soft or soft-ripened cheese; grows at refrigeration temperatures; survives in Cheddar and Colby, multiplies in cheeses such as Brie and Camembert.

- Enteropathogenic *Escherichia coli*: Involved in soft-ripened cheese outbreaks. Very virulent. Also verotoxigenic (enterohemorrhagic) *E. coli* (eg, *E coli* O157:H7); important emerging pathogen.

Medium Risk

- *Streptococcus* (Group A): Can cause mastitis; very virulent; survives in cheese.
- *Streptococcus* (Group C): Caused milkborne outbreaks. Can cause mastitis.
- *Brucella abortus*: Can cause mastitis; relatively heat insensitive, but eliminated by pasteurization.
- *Mycobacterium bovis*: Can cause mastitis; relatively heat insensitive, but eliminated by pasteurization.
- *Pseudomonas aeruginosa*: Opportunistic pathogen.
- *Coxiella burnetii*: Can cause mastitis; relatively heat insensitive, but eliminated by pasteurization.
- *Vibrio* species: Potential pathogen.
- *Aeromonas hydrophila*: Potential pathogen; predominantly aquatic.

Low Risk

- *Staphylococcus aureus*: Control measures effective and well-known.
- *Clostridium botulinum*: Control measures effective and well-known.
- *Clostridium perfringens*: No known outbreaks associated with cheese.
- *Corynebacterium diphtheriae*: No known outbreaks associated with cheese.
- *Bacillus cereus*: No known outbreaks in cheese.
- *Campylobacter jejuni*: No known outbreaks in cheese. Apparently does not survive in cheese.
- Viruses—hepatitis A, polio, retrovirus, coxsackie, adenovirus, herpes, oncogenic, foot and mouth disease: Known to contaminate milk. Transmission modes and heat resistance not understood.

Of the high risk pathogens, *Listeria monocytogenes* (though known in veterinary and medical bacteriology for decades) can be regarded as a recently emerged pathogen in dairy microbiology due to prolonged ripening and storage of cheeses at low temperatures (around 5°C), which favors proliferation of this psychrotrophic species. Because the species is quite ubiquitous, occurring in plant material, silage, raw milk, feces, and meat, it enters the cheese factory not only with raw milk but with diverse organic material. Especially soft and semi-soft cheeses with a bacterial or mold surface microflora (smear) are sensitive to recontamination and proliferation. *Listeria monocytogenes* must be absent from the environment of cheeses (vat, brine, cheese brands, smear, flavor, sinks, wells) and the cheese factory in order to produce a *Listeria monocytogenes*–free cheese.

Spahr and Url[124] have compiled data (Tables 23-15 and 23-16) on the experimental behavior of *L. monocytogenes* during cheese manufacturing and cheese ripening. In general, *Listeria* does not grow during the ripening period in semi-hard and semi-soft cheese without smear ripening, and numbers tend to decrease. The final *L. monocytogenes* concentration is in the same order of magnitude as in the initial milk. In smear-ripened cheese, however, a marked increase in *Listeria* counts occurs on the surface of contaminated cheeses. *L. monocytogenes* was reported by Ryser and Marth[115] to multiply in the interior of Camembert cheese. It should be noted that the survival time for *Listeria* was less than 1 day in experimental production of hard cheeses (Emmental, Gruyère) with cooking temperatures well above 50°C. In Parmesan, *Listeria* is dead before the official end of the ripening period. If a cheese like mozzarella is pasteurized after fermentation, *Listeria* are destroyed. All other cheese types such as semi-hard cheeses have rather long survival times for *Listeria* and must not be contaminated during manufacture and ripening.

Salmonella viable counts, like those of other pathogens increase by about one order of magnitude during cheese manufacture, but decrease again during cheese ripening (Table 23-17). Die-off is slower at the low ripening temperatures. These do not permit proliferation of *Salmonella* on the cheese surface or the interior.

In general, *E. coli* counts show a remarkable increase during the manufacture and ripening of cheese (up to three orders of magnitude). Finally, a decrease is observed (Tables 23-18 and 23-19).

In hard cheese, *Yersinia enterocolitica* is able to survive for periods of more than 56 days, whereas in feta cheese its behavior is severely influenced by the pH value of the cheese demonstrating an acid sensitivity (Tables 23-18 and 23-19).

Campylobacter jejuni is likely to be destroyed rapidly once the pH value decreases below 5.0 as in cottage cheese. No survival was observed in cheddar cheese after 15 days of ripening.

Brucella abortus counts decrease during the manufacture of cheese as well as during subsequent

Table 23-15 Behavior of *Listeria monocytogenes* during the First 24 Hours of Cheese Manufacture

		Cooking			*Log cfu ml^{-1}, g^{-1}*	
Cheese	*Milk*	*Temp. (°C)*	*Time (min)*	*pH Value: 24 Hours*	*0 Hours (milk)*	*24 Hours*
Hard cheese						
Cheddar	past.	38.9	90	NA	2.7	3.0
Colby	past.	37.8	45	NA	2.4–3.5	3.5–4.4
Brick	past.	38.9	40	5.3	2.2–3.1	3.0–4.7
Parmesan	past.	51	45	NA	4.5–5.5	5.0–6.2
Swiss	past.	50	30–40	5.2	4.2–4.5	4.1–4.4
Emmental	raw	53	45	5.2–5.4	4.6	< 0
Gruyere	raw	56	25	5.2–5.4	4.6	< 0
Semi-hard cheese						
Semi-hard	past.	32	NA	5.1–5.8	3.6–5.3	3.3–6.3
Semi-soft	raw	37	40	5.5	5.7–6.3	NA
Gouda	past.	35.5	NA	5.5	2.7	4.1–4.4
Maasdam	past.	35.5	NA	5.5	2.7	4.2
Trappist	past.	39	45	5.0–5.4	2.5–5.4	NA
Soft cheese						
Blue	past.	35–36.5	50	4.7–5.0	3.0–3.3	3.9–5.0
Camembert	past.	33	50	4.6	2.6–2.9	3.1–3.7
Mold-ripened soft	NA	NA	NA	4.9	2.0	2.0
Smear soft	NA	NA	NA	4.9	2.0	2.0
Other						
Mozzarella	past.	40/58–65[a]	30	5.2–5.3	4.0–5.0	< 0

Note: NA = Data not available.

[a] Temperature during stretching of curd in hot water.

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ripening. Survival time from an inoculum of 10^4 cfu ml^{-1} of milk was 6 days in hard cheeses and 10 to 17 days in soft cheese.

Staphylococcus aureus (besides *Salmonella*) has been the most prominent food poisoning agent in raw milk and recontaminated pasteurized milk cheeses because of the following reasons:

1. Contamination of raw milk during milking from the udder surface is inevitable.
2. In the case of *S. aureus*-induced mastitis, the bacteria are shed into the milk.
3. *S. aureus* is well adapted to a low water activity, eg, in salted cheese.
4. *S. aureus* is able to tolerate moderate lactic acid concentration as present in cheese.
5. *S. aureus* can multiply to high numbers during cheese manufacture and ripening in soft and semi-hard cheeses. In hard cheeses (Tables 23-20 and 23-21), a trend to survival, but decrease during ripening is evident.
6. *S. aureus* can produce enough enterotoxins in cheese to cause acute food poisoning.
7. *S. aureus* enterotoxin survives for months in cheese even if the viable counts decrease.

The only safe measure to avoid *S. aureus* in cheese is the use of pasteurized milk and control of post-pasteurization recontamination. Although *S. aureus* food poisoning is not a life-threatening disease, people feel very sick and will most likely not buy the incriminated cheese again.

Microbiology of the Cheese Surface

Like fermentation in the cheese interior, the microbiological processes occurring at the surface of cheese during manufacture, ripening, and storage of cheese must be considered as a solid-state substrate fermentation. The problem starts with the definition of the surface (only the microbial biofilm or in addition the uppermost 1 to 5 mm of the solid substrate?). In order to describe the entire system (biofilm and substrate), a practicable definition of the cheese surface is proposed as the uppermost layer of cheese (up to 5 mm) including the surface biofilm. By this definition, it is possible to describe the dynamic changes of the sur-

Table 23-16 Behavior of *Listeria monocytogenes* during Cheese Ripening and Storage

		Storage		*pH Value*		*Log cfu g-1*			
Cheese	*Milk*	*Temp. (°C)*	*Time (days)*	*24 Hours*	*Final*	*24 Hours*	*Maximum*[a]	*Final*	*Survival Time (days)*
Hard cheese									
Cheddar	past.	6/13	434	NA	5.0–5.1	3.0	3.7	1.0–3.0	154 to > 434
Colby	past.	4	140	NA	5.0–5.2	3.5–4.4	4.7	2.3–4.0	> 140
Brick	past.	15/10	154–168	5.3	5.6–6.2	3.0–4.7	5.5[b]	4.3–4.8[b]	> 168
Parmesan	past.	12.8	335–349	NA	5.0–5.1	5.0–6.2	4.5	< 1.0	14–112
Swiss	past.	7/24	10/80	5.2	5.2–5.4	4.1–4.4	4.1–4.4	< 0	< 66 to < 80
Emmental	raw	23/12	120	5.2–5.4	5.8	< 0	< 0	< 0	< 1
Gruyere	raw	16/14	120	5.2–5.4	5.6–5.7	< 0	< 0	< 0	< 1
Semi-hard cheese									
Semi-hard	past.	15	60	5.1–5.8	5.1–5.8	3.3–6.3	5.3	2.8–5.3	> 60
Semi-soft	raw	12	126	5.5	6.5	NA	6.9	6.7	0 to > 126
Gouda	past.	13	48	5.5	NA	4.1–4.4	4.4	3.2–3.9	> 48
Maasdam	past.	13	48	5.5	NA	4.2	4.2	3.6	> 48
Trappist	past.	16–18/10	95	5.0–5.4	4.7–5.1	NA	6.8	2.7–5.6	> 90
Soft cheese									
Blue	past.	9–12/4	120	4.7–5.0	5.7–6.2	3.9–5.0	4.8	1.0–2.2	> 120
Camembert	past.	15–16/6	66	4.6	7.0–7.7	3.1–3.7	7.2	4.2–7.2	> 66
Mold soft	NA	16.5/5	91	4.9	7.1–7.7	2.0	7.2	3.4–7.2	> 91
Smear soft	NA	17/5	91	4.9	5.9–7.4	2.0	6.0	< 1–6.0	> 91
Other									
Mozzarella	past.		NA	5.2–5.3	NA	< 0	NA	NA	< 1

Note: The experiments are a continuation of those in Table 23-15.

NA = Data not available.

[a] Maximum in any trial.

[b] Log counts on surface of cheese were 6.5–6.9 (max) and 5.1–6.5 (final).

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face microflora and the resulting biochemical changes in the cheese matrix.

The traditional use of spontaneous surface fermentation has been replaced by the use of specific starter cultures (molds, yeasts, and coryneform bacteria), which are either added to the cheese milk or applied during ripening by regular smearing of the cheese surface with a proper bacterial/yeast culture.

Surface of Brick Cheeses. It is generally assumed that initially a yeast microflora has to degrade some of the lactic acid of the primary lactic fermentation in order to allow growth of the more neutrophilic coryneform bacteria. A recent investigation of surface-ripened brick cheeses (Limburger, Romadour, Tilsiter, Münster, Weinkäse, and Harzer[140]) using undefined ripening cultures found *Debaryomyces hansenii* and *Galactomyces geotrichum* prevalent in rennet cheeses (Table 23-22), and *Kluyveromyces marxianus* and *Pichia membranaefaciens* in acid-cured cheese. The coryneform bacteria that followed were identified as *Arthrobacter nicotianae, Brevibacterium linens, Corynebacterium ammoniagenes, Corynebacterium variabilis*, and *Rhodococcus fascians* (Table 23-23). However, about one-third of the isolated strains could not be determined down to the species level. It is hoped to develop from these cultures a suitable starter culture.

Another problem of these surface-ripened cheeses is the growth of Gram-negative bacteria of probable enteric origin if raw milk was the substrate of cheese making. However, if pasteurized milk is used, lactose-positive Gram-negative bacteria will still be present. Presumably, this is due to post-processing contamination. Figure 23-10 shows the development of the surface microflora of a soft cheese made from pasteurized milk with a defined yeast/*Brevibacterium* starter culture. The flora grows up from a low number to take over the dominant role. The Gram-negative bacteria identified out of 169 isolates in-

Table 23-17 Behavior of *Salmonella* during Cheese Ripening and Storage

Cheese	*Milk*	*Storage*		*pH Value*		*Log cfu g^{-1}*			*Survival Time (days)*
		Temp. (°C)	*Time (min)*	*24 Hours*	*Final*	*24 Hours*	*Maximum*[a]	*Final*	
Hard cheese									
Cheddar, Colby	past.	0–10	NA	5.2–5.3	5.1–5.4	4.8[b]	NA	< 0	60–270
Cheddar	raw	5	NA	NA	5.1	2/11[c,d]	NA	< 0	< 30 to > 135
Manchego	past.	10	70	5.2	NA	5.5–5.5[e]	NA	< 0	28 to < 56
Monterey Jack	past.[f]	4.5	183	NA	NA	4.0–6.5[g]	8.0	< 0–5.5	30 to > 183
Other									
Domiati	past.	30	39	NA	NA	2.3–4.5[h]	NA	< 0	15–36

Note: NA = Data not available.

[a] Maximum in any trial.

[b] *S. senftenberg 775W, S. typhimurium TM1, S. new brunswick* 1608, *S. newport.*

[c] Milk naturally contaminated with *S. müenster.*

[d] In 2 of 11 vats *Salmonella* survived.

[e] *S. typhi, S. enteritidis, S. typhimurium.*

[f] Whole milk and ultrafiltered milk.

[g] *S. typhimurium.*

[h] *S. typhi.*

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Table 23-18 Behavior of Other Gram-Negative Bacteria during the First 24 Hours of Cheese Manufacture

Cheese	*Milk*	*Cooking*		*pH Value: 24 Hours*	*Log cfu ml^{-1}, g^{-1}*	
		Temp. (°C)	*Time (min)*		*0 Hours (milk)*	*24 Hours*
Pathogenic *Escherichia coli*						
Hard cheese						
Colby-like	past.	38.9–4.0	60	4.9–6.5	2.0–3.0	3.3–6.0
Brick	past.	36.5	45	5.2	2.7	5.2–5.6
Yersinia enterocolitica						
Hard cheese						
Colby-like	past.	40	60	5.0–5.3	3.0	3.5–6.0
Other						
Feta	past.	37	20–30	4.3–5.0	2.7–7.3	4.3–8.2
Feta[a]	past.	37	20–30	5.0–6.3	2.7–6.7	6.8–9.3
Campylobacter jejuni						
Hard cheese						
Cheddar	past.	38.8	60	NA	3.0–6.4	2.9–6.9[b]
Other						
Cottage	past.	55	30	4.5	6.0–6.2	<1

Note: NA = Data not available.

[a] Slow acid production.

[b] Curd, after salting, before pressing.

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Table 23-19 Behavior of Other Gram-Negative Bacteria during Cheese Ripening and Storage

		Storage		*pH Value*		*Log cfu g^{-1}*			
Cheese	*Milk*	*Temp. (°C)*	*Time (days)*	*24 Hours*	*Final*	*24 Hours*	*Maximum*[a]	*Final*	*Survival Time (days)*
Pathogenic *Escherichia coli*									
Hard cheese									
Colby-like	past.	3/10	91	4.9–6.5	5.2–5.7	3.3–6.0	6.0	< 0 to 3.0	28–84
Brick	past.	7	49	52	5.2	5.2–5.6	NA	2.8–4.3	< 49
Yersinia enterocolitica									
Hard cheese									
Colby-like	past.	3	56	5.0–5.3	5.0–5.3	3.5–6.0	NA	< 0 to 2.0	< 49 to > 56
Other									
Feta	past.	22	5	4.3–5.0	4.4–4.5	4.3–8.2	8.2	< 0	< 5
Feta[b]	past.	22/4	30	5.0–6.3	4.9–5.3	6.8–9.3	9.3	4.2–8.5	> 30
Campylobacter jejuni									
Hard cheese									
Cheddar	past.	7	60	NA	NA	2.9–6.9[c]	NA	< 1	15 to < 30
Other									
Cottage	past.	4	NA	NA	NA	< 1	NA	< 1	< 1

Note: The experiments are a continuation of those in Table 23-18. NA = Data not available.

[a] Maximum in any trial.

[b] Slow acid production.

[c] Curd, after salting, before pressing.

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Table 23-20 Behavior of *Staphylococcus aureus* during the First 24 Hours of Cheese Manufacture

		Cooking			*Log cfu ml^{-1}, g^{-1}*	
Cheese	*Milk*	*Temp. (°C)*	*Time (min)*	*pH Value: 24 Hours*	*0 Hours (milk)*	*24 Hours*
Hard cheese						
Cheddar unsalted	past.[a]	NA	NA	5.0–5.3	2.9–4.6	6.4–7.6
Cheddar salted	past.	NA	NA	5.6–6.4	2.9–4.6	7.5–8.0
Manchego	past.	32–33	NA	5.5–5.6	4.6–5.7	4.6–5.2
Manchego	raw	30	15	4.9	3.0–5.0	6.1
Manchego	raw	36–40	15	5.0–5.1	3.0–5.0	6.2–6.3
Semi-hard cheese						
Gouda	past.[b]	NA	NA	5.1–5.9	2.9–7.6	5.9–8.6
Other						
Domiati	raw	NA	NA	5.2	3.8–5.3	6.4–7.5

Note: NA = Data not available.

[a] Pasteurized.

[b] Or bactofuged.

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Table 23-21 Behavior of *Staphylococcus aureus* during Cheese Ripening and Storage

		Storage		*pH Value*		*Log cfu g^{-1}*			
Cheese	*Milk*	*Temp. (°C)*	*Time (days)*	*24 Hours*	*Final*	*24 Hours*	*Maximum*	*Final*	*Survival Time (days)*
Hard cheese									
Cheddar unsalted	past.	4	42	5.0–5.3	5.0–5.3	6.4–7.6	6.5	4.0–5.6	> 42
Cheddar unsalted	past.	11	42	5.0–5.3	5.0–5.3	6.4–6.7	6.9	2.4–4.9	> 42
Cheddar salted	past.	4	42	5.6–6.4	5.4–6.3	7.5–8.0	7.9	6.2–7.6	> 42
Cheddar salted	past.	11	42	5.6–6.4	5.3–6.2	7.5–8.0	8.5	6.0–8.1	> 42
Manchego	past.	15	58	5.5–5.6	5.3–5.5	4.6–5.2	6.7	< 0	< 58
Manchego	raw	5	60	5.1	5.1	6.2	NA	3.3	> 60
Manchego	raw	10–20	60	5.1	5.1–5.3	6.2	NA	1.3–2.3	> 60

Note: The experiments are a continuation of those in Table 23-20. NA = Data not available.

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cluded 23% *Klebsiella oxytoca,* 7% *Klebsiella pneumoniae,* 1% *Klebsiella ozaenae,* 19% *Serratia liquefaciens,* 4% *Citrobacter freundii,* 7% *Enterobacter agglomerans,* and 3% *Enterobacter cloacae.* Of the isolates, 29% could not be identified with the API 20E system.

A preliminary analysis of 18 other surface-ripened cheese types including Appenzeller, Tilsit, Tête de Moine, Romadour, and others showed a similar Gram-negative surface flora. However, prominent species on the surface of some semi-hard cheeses were *Proteus vulgaris, P. rettgeri, P. stuarti, Morganella morganii,* and *Hafnia alvei.* Obviously, surface-ripened cheeses do contain Gram-negative bacteria. Their interpretation as fecal coliforms is questionable because many of these species also occur in other environments including soil, water, and plants. The fact that some of them are regarded as opportunistic pathogens has to be considered if a diet for immuncompromised persons is proposed.

Surface of Mold-Ripened Cheeses (Camembert and Brie[110]). The main hurdles regarding microbiological safety of cheeses are their water activities and their pH values, ie, their organic acid contents. These hurdles are high in hard cheeses, but tend to disappear in soft cheeses during ripening (Table 23-11). The surface flora of *Penicillium camemberti/caseicolum* developing together with certain yeasts during the first week of ripening leads to a degradation of lactic acid. Together with proteolysis and the resulting products (peptides, amino acids, NH_3), this

Table 23-22 Occurrence of Yeast Species on Brick Cheese Varieties Limburger, Romadour, and Weinkäse

	Dairy:	*A*	*A*	*A*	*B*	*C*	*C*	*C*	*C*	*D*	*D*
	Cheese Type:	*Limburger*	*Limburger*	*Weinkäse*	*Weinkäse*	*Limburger*	*Limburger*	*Limburger*	*Romadour*	*Romadour*	*Romadour*
Species	*Fat Content %:*	*40*	*40*	*45*	*45*	*20*	*40*	*45*	*50*	*20*	*50*
Candida catenulata					2	1	12		3		
Candida intermedia					2			11		56	10
Debaryomyces hansenii		55	65	86	95	49	85	54	69		
Galactomyces geotrichum		21	17	4	1	50	2	31	6	44	3
Torulaspora delbrueckii		24									
Trichosporon beigelii				3					22		
Yarrowia lipolytica			19					4			87

Note: Numbers are given as percent of total yeast count.

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Table 23-23 Occurrence of Coryneform Bacteria on Brick Cheese Varieties Limburger, Romadour, Weinkäse, Harzer, and a Special Soft Cheese of 2 kg Weight

	Dairy:	*A*	*B*	*C*	*D*	*E*	*F*
	Cheese Type:	*Limburger and Weinkäse*	*Weinkäse*	*Limburger and Romadour*	*Romadour*	*Harzer*	*Soft Cheese*
Species	*Fat Content %:*	*40–45*	*45*	*20–50*	*20–50*	*< 2*	*40*
Arthrobacter citreus		1					
Arthrobacter nicotianae		5	5	1	2	1	
Arthrobacter spp.		5	7		2	2	5
Brevibacterium fermentans						1	2
Brevibacterium linens		3	2	4	5	2	9
Brevibacterium oxidans							1
Corynebacterium ammoniagenes		8	16	3	3	1	5
Corynebacterium spp.		4	2	3	1		3
Corynebacterium variabilis		1	1			6	4
Microbacterium spp.							2
Microbacterium imperiale							5
Rhodococcus fascians							15

Note: Numbers of the 148 identified are given.

Source: Reprinted with permission from N. Valdes-Stauber, N. Scherer, and H. Seiler, Identification of Yeasts and Coryneform Bacteria from the Surface Microflora of Brick Cheese, *International Journal of Food Microbiology,* Vol. 34, pp. 115–129, © 1997, Elsevier Applied Science.

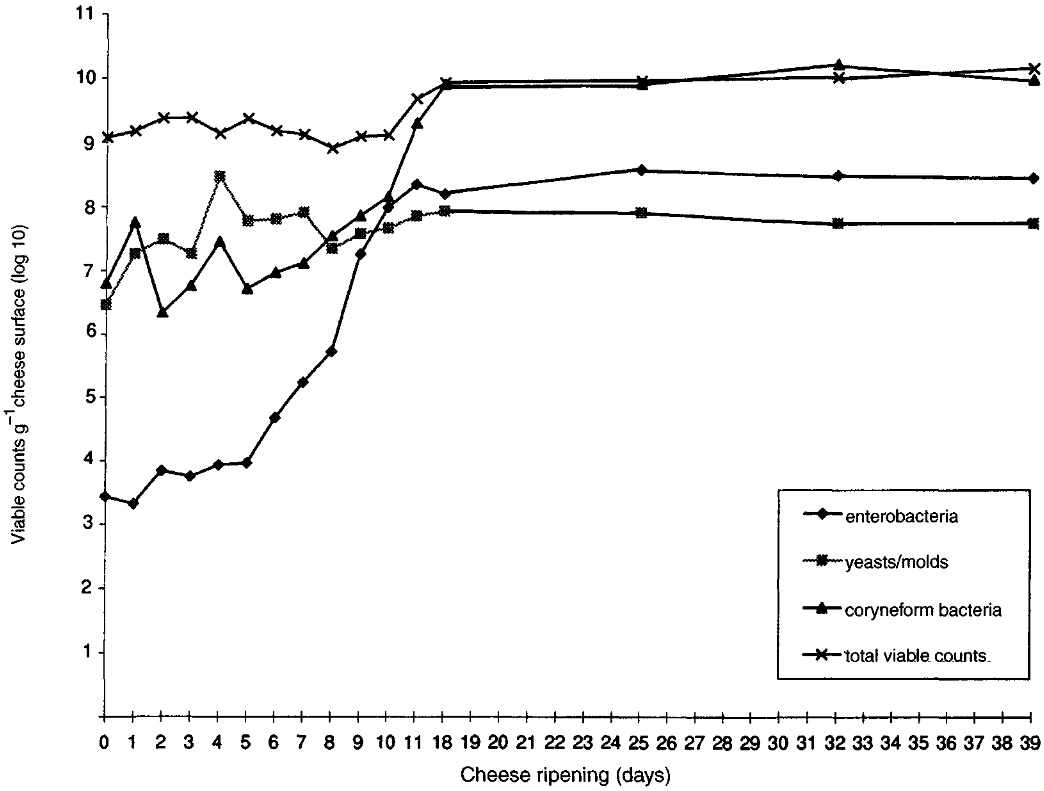

Figure 23-10 Development of the Surface Microflora of an Experimental Red Smear Soft Cheese Made from Pasteurized Milk and Recontaminated with Enterobacteria. Courtesy of S. Gianotti, A. Burri, and M. Teuber, Eidgenössische Technische Hochschule, Zurich, Switzerland.

induces an increase of the surface pH values from about 4.5 right after the end of the lactic fermentation to well above 7, and to about 6 in the cheese interior after 3 to 4 weeks of ripening.[78] Prolonged ripening of 60 days may produce an interior pH of >7.[115] Because the water activities of mold-ripened cheeses like Camembert and Brie (a_w 0.97 to 0.98) are also high, there is little or no barrier to prevent growth of undesired microbes if they should be present due to primary (raw milk, see below) or secondary contamination during manufacture. Because raw milk cannot be produced with the necessary low microbiological risk, many countries prescribe that pasteurized milk must be used for the production of soft cheeses. The prominent listeriosis outbreaks from cheese in Europe were all caused by contaminated soft cheeses made with unpasteurized milk (Table 23-14). Recent microbiological analysis of European soft cheeses made from raw milk demonstrated that about 50% of them were contaminated with *Listeria* and/or *Escherichia coli*.[41] Another hazard of raw milk soft cheeses is the presence of antibiotic-resistant enterococci and staphylococci. The Gram-negative microflora of such soft cheeses is very similar to the one described above for surface-ripened brick cheeses.[94] Although pasteurization of milk eliminates foodborne pathogens, a low level of Gram-negative bacteria can only be obtained with cheeses made from pasteurized milk if post-pasteurization recontamination, eg, by the cheese makers, the process water, the rennet preparation, or the equipment is prevented.

A recent analysis[30] of raw milk from 27 selected farms delivering milk for raw milk Camembert production revealed that the predominant bacteria were *Pseudomonas* (2000 cfu ml^{-1}), lactococci (760 cfu ml^{-1}), and Micrococcaceae (720 cfu ml^{-1}). Lactic acid bacteria were detected in all samples. Coliforms were present in most samples, but 84% had counts below 100 cfu ml^{-1}. *Staphylococcus aureus* was detected in 62% of milks (average 410 cfu ml^{-1}), *Escherichia coli* in 80% (less than 10 cfu ml^{-1}), *Clostridium perfringens* in 100% (but less than 1 cfu ml^{-1}). During 6 months, 2 of the tested milk samples were positive for *Salmonella* (2.9%), 4 for *Listeria monocytogenes* (5.8%), 25 for *Yersinia enterocolitica* (36%, the proportion of pathogenic strains being probably low), and 1 for *Campylobacter* (1.4%). Because the milks of different farms are mixed in cheese factories, even a low percentage of contaminated milk samples will spoil the whole lot used for raw milk cheese production. This means that the seemingly low risks on a farm basis are unpredictably multiplied by the practice of bulking milk. These investigations, in this author's eyes, are a further strong argument for the use of pasteurized milk for soft and even semi-hard cheese production.

23.7.3 Biogenic Amines in Fermented Dairy Products and Cheeses

Biogenic amines are typical metabolites found in fermented food and fodder[9] including beer, wine, cheese, fermented raw sausage and meat, and silage. They are the decarboxylation products of amino acids released from food proteins due to enzymatic hydrolysis, eg, during cheese ripening. In cheese, it is mainly the contaminating or autochthonous microflora, and not the starter culture, that has high decarboxylating activities. Active bacteria in this respect are certain lactobacilli like *Lactobacillus buchneri*, pediococci, and enterococci. Special attention should be directed to the Gram-negative microflora of the cheese surface (*Serratia, Klebsiella, Enterobacter,* and *Proteus* species) with a high potential for putrescine and cadaverine production in addition to histamine and tyramine. Raw milk products with a long ripening time are especially prone to develop high amine concentrations (Table 23-24).

There is no general information about the quantity of biogenic amines a person has to consume to show typical symptoms of amine poisoning. The reported examples of such outbreaks due to cheese are scarce and involve one each in 1967, 1976, 1977, 1980, and 1994 in Holland (Gouda), California (Swiss cheese), France (Ceshire), Canada (Cheddar), and Germany (Emmental), respectively, and include cheeses made from raw as well as from pasteurized milk. In the case of the Emmental, the remaining cheese was found to contain 619 mg histamine, 456 mg tyramine, 1500 mg cadaverine, and 424 mg putrescine kg^{-1}. An outbreak with 38 cases in Washington (Swiss cheese) in 1976 was obviously due to histamine levels well above 1000 mg kg^{-1}, and an outbreak in New Hampshire (Swiss cheese) was caused by similar histamine levels. In summary, the presence of amines in cheeses has to be expected. However, the actual levels depend on the type of cheese and the chances of secondary microbial contamination, in the case of cheese made from pasteurized milk, or the endogenous microflora of raw milk in the case of cheeses made from raw milk. Judging from the low number of cases reported in the literature, the risk of amine poisoning due to consumption of cheese is low. The investigated starter cultures of lactic acid bacteria used by the dairy industry have no potential to form biogenic amines.[129]

Table 23-24 Histamine, Tyramine, Cadaverine, Putrescine, β-phenylethylamine, and Tryptamine Content of European Cheese and Fermented Milk Products

Product	*Histamine*	*Tyramine*	*Cadaverine*	*Putrescine*	*β-Phenylethylamine*	*Tryptamine*
1.1. Saanen	(169)	(26)				
1.2. Sbrinz	4 (6)	0 (5)				
1.3. Parmiggiano Reggiano	34 (56)	154 (841)	63 (252)	31 (85)		
1.4. Grana Padrano	125 (137)	7 (8)				
2.1. German Mountain cheese	335 (885)	207 (603)	79 (349)	85 (377)		
2.2. Swiss Mountain cheese	17 (42)	116 (221)	3 (6)	33 (52)		
2.3. German Emmentaler	375 (1062)	111 (674)	51 (178)	37 (294)	4 (55)	
2.4. Ground Emmentaler	466 (916)	225 (565)	415 (1739)	175 (664)		
2.5. Swiss Emmentaler	34 (105)	138 (368)	2 (4)	1 (2)		
2.6. Emmentaler from pasteurized milk	171 (966)	135 (670)	60 (208)	20 (63)		
2.7. Appenzeller	244 (567)	238 (702)	247 (542)	26 (111)		
2.8. Greyerzer	10 (32)	6 (15)	17 (22)	6 (26)		
3.1. Cheddar	370 (850)	11 (32)	30 (144)	7 (232)		31 (300)
3.2. Edam	72 (348)	100 (84.5)	57 (184)	31 (184)		
3.3. Gouda	98 (409)	119 (1064)	68 (361)	52 (325)		
3.4. Tilsit	79 (198)	235 (658)	380 (1091)	115 (485)		
4.1. Blue cheese	23 (95)	92 (601)	25 (112)	13 (81)		
4.2. Soft cheese with red smear	23 (85)	102 (46)	223 (957)	58 (346)		
4.3. Soft cheese with *Penicillium camemberti*	24 (119)	16 (159)	42 (490)	31 (237)		
4.4. Raw milk soft cheese	33 (242)	161 (1457)	211 (957)	74 (346)	6 (93)	
5. Handkäse	105 (225)	592 (1077)	1289 (2905)	740 (1284)		
6.1. Sour cream	(1)	(10)				
6.2. Butter milk	(0.7)					
6.3. Sour milk	(1.2)					
6.4. Yogurt	(13)	(8.5)				

Note: Mean values are given in mg kg^{-1}. Maximum observed amounts are recorded in brackets. (1) extra hard cheese, (2) hard cheeses, (3) semi-hard cheeses, (4) soft cheeses, (5) sour milk cheese, (6) fermented milk products.

Source: Adapted with permission from G. Kielwein, Biogene Amine in Lebensmittein, Milch und Milchprodukte, in *Biogene Amine in der Ernährung*, D.M. Beutling, ed., pp. 113–129, © 1996, Springer Verlag.

23.7.4 Antibiotic-Resistant Bacteria in Cheese

Antibiotic-resistant bacteria are becoming an increasing threat to the treatment of almost all bacterial diseases of man and animals. Humankind is approaching a self-made or at least self-induced crisis in antibiotic resistance.[15,25,95,145] For several reasons, this may become also a problem for the food industry:

1. Food of animal origin (milk, meat, and eggs) is produced from antibiotic-treated animals. Antibiotics are routinely used, for example, to treat or prevent mastitis in dairy cows, to fight intestinal and respiratory infections in calves, pigs, and chickens, or to induce a nutritive effect with subtherapeutic levels of antibiotics. It is estimated that about half of the total amount of antibiotics produced worldwide is used in agriculture.[79]
2. Some of the bacteria occurring in food and hitherto regarded as harmless contaminants have developed into important pathogens especially in nosocomial (hospital-induced) infections, including enterococci, coagulase-negative staphylococci (CNS), and certain opportunistic enterobacteria like *Klebsiella, Enterobacter, Proteus,* and *Serratia* species. Such bacteria are not uncommon in raw milk cheeses and the cheese surface flora (see above).
3. Selection and proliferation of antibiotic-resistant bacteria in the food-producing animals and the raw material derived from them may lead to direct contamination or recontamination in the

case of nonpasteurized or unheated raw material (milk for cheeses, meat for sausages) or pasteurized or thermized products, respectively.

4. Transfer of antibiotic resistances, via conjugative plasmids or transposons, between members of the microflora of food and potentially pathogenic bacteria has recently been documented *in vitro*.[137]

For example, multiply resistant enterococci and staphylococci have been isolated without difficulty from raw milk cheeses and raw cured sausages (Table 23-25). Enterococci numbered between 10^2 and 10^7 g^{-1} in cheeses and between 10^2 and 10^5 in sausages, staphylococci between 10^2 and 10^5 in cheeses, and 10^3 and 10^6 in sausages, respectively. Antibiotic resistances included resistance to penicillin, cefalotin, chloramphenicol, kanamycin, streptomycin, gentamicin, tetracyclines, fucidin, erythromycin, rifampicin, lincomycin, vancomycin, and norfloxacin in enterococci and resistance to erythromycin, tetracycline, gentamicin, chloramphenicol, rifampicin, penicillin, neomycin, and lincomycin in staphylococci. About 15% of the isolated enterococcal strains could transfer their antibiotic resistances to other Gram-positive species with the aid of conjugative plasmids and transposons. Some characteristic resistance genes were identified on a molecular level to be similar or identical with genes known from pathogenic human strains (eg, *tetM, tetS, tetK, ermAM, linA,* cat_{pIP501}).

Table 23-25 Distribution of Antibiotic Resistances of Antibiotic-Resistant Enterococci Isolated from 56 Cheeses in 1980–81 (15 strains), in 1991 (12 strains), and 1995 (29 strains)

Antibiotic	*Number of Resistant Strains*
Gentamicin	45
Tetracyclin	33
Erythromycin	27
Chloramphenicol	18
Penicillin	10
Cefalotin	8
Fucidin	8
Furadoin	5
Rifampicin	4
Vancomycin	4

Note: All cheeses were purchased as raw milk cheeses from different parts and countries in central Europe.

Source: Reprinted with permission from M. Teuber, V. Perreten, and F. Wirsching, Antibiotikumresistente Bakterien: Eine Neue Dimension in der Lebensmittelmikrobiologie, *Lebensmittelwissenschaft und Technologie,* Vol. 29, pp. 182–198, © 1996, Frieden Verlag.

It should be mentioned that antibiotic-resistant enterococci were normally not detected in cheeses known to be made from pasteurized milk. However, if enterococci are detected in cheeses made from pasteurized milk, this is a matter of post-pasteurization contamination, or of high levels of enterococci ($>10^4$ cfu ml^{-1}) in milk before pasteurization (D-value around 2 minutes at 65°C, z-value up to 8°C), or of the use of starter cultures containing enterococci.

An interesting antibiotic-resistant *Lactococcus lactis* subsp. *lactis* strain was isolated from a raw milk Brie cheese (10^8 cfu g^{-1}). This strain was resistant to chloramphenicol, tetracycline, streptomycin, and norfloxacin. The resistance genes were localized on a 30 kilobase plasmid, which was completely sequenced.[106] This composite plasmid had 29 open reading frames: 15 ORFs including 6 IS elements had to do with DNA rearrangement, regulation, mobilization, and replication, 7 with antibiotic resistance, and 7 are still unknown, ill defined, or truncated. In this plasmid, *Lactococcus lactis* K214 has, with the help of IS elements, collected genetic information from four other species *(Listeria monocytogenes, Enterococcus faecalis* and *faecium, Staphylococcus aureus)* as evidenced by the high DNA homology of the sequenced genes with known genes from clinical isolates of such bacteria (Figure 23-11). The data so far collected demonstrate the presence of transmissible antibiotic-resistant genes in bacteria in raw milk cheeses (and in pasteurized cheeses if recontaminated!). The use of enterococci as starter cultures should be further questioned on the basis of the high gene-exchange potential of these bacteria irrespective of whether they are pathogenic or not. As a consequence, if we want to minimize the spread of antibiotic-resistant bacteria in the human population, food products containing antibiotic-resistant bacteria have to be banned generally from consumption, and specifically for high-risk parts of the population. In the case of cheeses, the use of pasteurized milk is an alternative to avoid such problems if recontamination of the treated milk after pasteurization is prevented.

23.8 CONCLUDING REMARKS

Modern fermented dairy products (sour milk, yogurt, and cheeses) have directly evolved from traditional, spontaneously fermented commodities that are part of the human cultural inheritance. The scientific exploration of the responsible microorganisms has resulted in a clear taxonomic differentia-

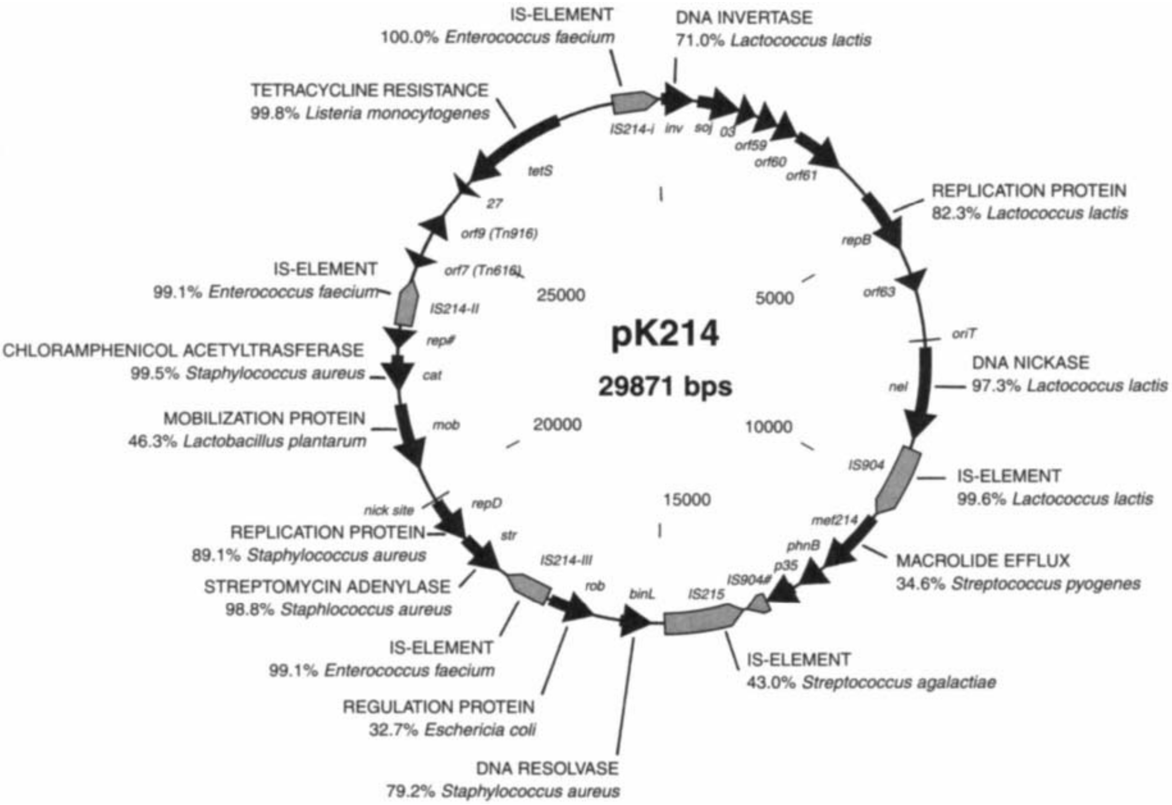

Figure 23-11 Molecular Structure of an Antibiotic-Resistant Plasmid from *Lactococcus lactis* subsp. *lactis.* The plasmid was detected in strain K214 isolated from a raw milk Brie. Its complete nucleotide sequence revealed that the resistance genes are almost identical with genes previously described in pathogenic bacteria like *Listeria monocytogenes* (tetracycline resistance) and *Staphylococcus aureus* (chloramphenicol and streptomycin resistance). The composite plasmid has obviously been assembled with the aid of insertion elements. The % data relate to amino acid identity of the corresponding proteins. *Source:* Data from V. Perreten, et al., Antibiotic Resistance Spread in Food, *Nature,* Vol. 389, pp. 801–802, © 1997.

tion of the technologically useful lactic acid bacteria, bifidobacteria, brevibacteria, propionibacteria, yeasts, and molds (*Penicillium camemberti* and *P. roqueforti*) from undesirable pathogenic and potentially pathogenic microbes like enterobacteria, staphylococci, listerias, *Brucella* spp., enterococci, and mycotoxin-producing fungi.

The biochemical reactions of starter cultures contributing to the typical properties of sour milk, yogurt, and cheeses are well characterized. Efficient and microbiologically safe starter culture preparations in liquid, freeze-dried, or deep-frozen, concentrated forms are provided in sufficient diversity by a responsible starter culture industry. One problem, however, not yet completely solved is the bacteriophage infection of lactic acid bacteria in cheese manufacture that cannot be performed with heat-sterilized milk. Whether genetically modified phage-resistant starter cultures developed by molecular biologists will be able to overcome the problem will depend on a complex set of issues including biosafety concerns, legal, and consumer acceptance.

The main hygiene issue is the use of raw milk for cheese making if the milk is contaminated with pathogenic bacteria that survive and eventually multiply during cheese making and ripening (*Salmonella, Brucella, Listeria monocytogenes, E. coli* O157, *Staphylococcus aureus,* and others). Likewise, recontamination of pasteurized or microfiltered milk and cheeses made from these substrates has been observed, but can be prevented by a proper hazard analysis critical control point (HACCP) system. A specifically high hygienic risk remains in raw milk soft cheese due to lack of hurdles (high a_w values and increased pH values during ripening). Recently, the presence of antibiotic-resistant pathogenic and potentially pathogenic bacteria (staphylococci and enterococci) in cheeses has been of increased concern.

The use of fermented dairy products as vehicles for probiotic bacteria (mainly certain lactobacilli and bifidobacteria) has gained economic importance. The claimed health effects for the normal healthy consumers remain to be established.

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24 Eggs and Egg Products

Ronald G. Board

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24.1 INTRODUCTION

Most eggs for commerce are laid by the domestic fowl, *Gallus gallus*. Three facets of egg and egg product research will be discussed in this chapter.

1. The rotting of eggs in the distribution chain led to extensive investigations of the course of microbial infection of the contents of shell eggs. Not only were the major causative agents of rots identified but also several components of the antimicrobial defense systems in eggs.
2. A progressive increase in the number of shell eggs used to make egg products over the past 50 years has focused attention on the link between the microbiological status of eggs and the microbiological quality of egg products, especially with respect to foodborne pathogens, particularly *Salmonella.* This trend and the importance of this link is likely to be accentuated in the next few years. It has been forecast[8] that approximately 50% of eggs produced in the United States (US) will be used to prepare egg products by the end of the century.
3. The recent "pandemic" associated with *Salmonella enteriditis* has drawn attention to the contribution that an intensive and vertically integrated poultry industry can make to the dissemination of a particular foodborne pathogen among humans. Studies of this topic have identified the inadequacy of our knowledge of the antimicrobial systems present in the oviduct of hens as well as of the changes that occur in stored eggs.

There has been a tendency for topics 1 and 3 above to be studied in an intellectual vacuum because the role of the egg in the breeding biology of birds has been ignored. Birds produce cleidoic (closed box) eggs. A hen lays down a food store (principally in the yolk), a reservoir of water (mainly in the albumen), and a depot of minerals (in the shell). The first two must be sufficient in amount and chemical composition to support embryogenesis and to sustain a chick for a short period after hatching. The embryo exchanges O_2 and CO_2 with the atmosphere via pores in the shell. An egg represents a huge investment of time and effort on the part of a hen. Obviously, natural selection must have endowed an egg with a defense of this investment against colonization by saprophytic microorganisms. Studies of the rotting process (see below) led to the recognition of several antimicrobial systems and the realization that their efficiency waned with time, and that they were negated by inappropriate treatment of eggs at any stage from oviposition onwards. If one assumes that the domestic fowl lays a clutch of 12 to 14 eggs before settling down to incubation and that it will take about 5 days for the yolk (the most vulnerable entity) to be fully enveloped in a cellular rather than a nonliving (fibrous) membrane, then it can be assumed that the first egg of a clutch would need an integrated antimicrobial system for about 20 days with storage at ambient temperature. As will be noted repeatedly in this chapter, microbiological problems in eggs tend to be manifested some 20 days post-oviposition.

24.2 THE FORMATION, STRUCTURE, AND COMPOSITION OF EGGS

For a detailed discussion of this topic, see Burley and Vadehra.[32] The yolk (water, 47.5%; lipid, 33.0%; and protein, 17.4% by weight) of the egg is laid down in an ovarian follicle, the bulk of the material being deposited over a 6 to 10 day period (Figure 24-1). On leaving the follicle, the yolk is enveloped in a fibrous membrane. Additional fibrous material as well as ovomucin, lysozyme, and other proteins of relatively small molecular weights are laid down on this membrane when the yolk enters the oviduct. At maturity, the yolk (vitelline) membrane is trilaminate, two fibrous layers being separated by a continuous membrane the structure and chemical composition of which are unknown. The yolk is encapsulated in oviductal proteins (Table 24-1) to form the egg white in the 4 hours it takes to pass along the oviduct, a tube—one end of which opens into the peritoneum and the other into the cloaca. The white (water, 88%; proteins, 11%; and free carbohydrate, 0.5% by weight of whole white) is enveloped eventually in material that forms the anastomizing fibers of the inner and outer shell membranes. These coaxial fibers have a core rich in elastin-like protein surrounded by a mantle of glycoproteins. The principal water reserve is laid down—in a process known as plumping—during, and for a short period following, the deposition of the shell membranes and the cone layers of the shell.

The outer shell membrane provides the foundation for the shell, which is built up over a period of about 20 hours. Calcium carbonate, as calcite in a polycrystalline state, is the major component. The shell is permeated with fibrous material formed from short peptides of hepatic origin. Pores (upwards of 17, 000 per shell), originate at the inner surface of the shell and terminate in a campanulate orifice at the outer (Figure 24-2). The cuticle, a relatively dense material rich in glycoproteins, is laid down on the surface of the shell. With a perfectly formed egg, the entire surface of the shell is covered with this material. At the time of lay the cuticle is a slippery, lustrous layer that dries rapidly on exposure to the atmosphere. Within a few minutes of laying, it dries out such that the shell is covered with a somewhat flakey, fissured layer and the outer orifice of a pore canal is crudely plugged with vesicular material.[24]

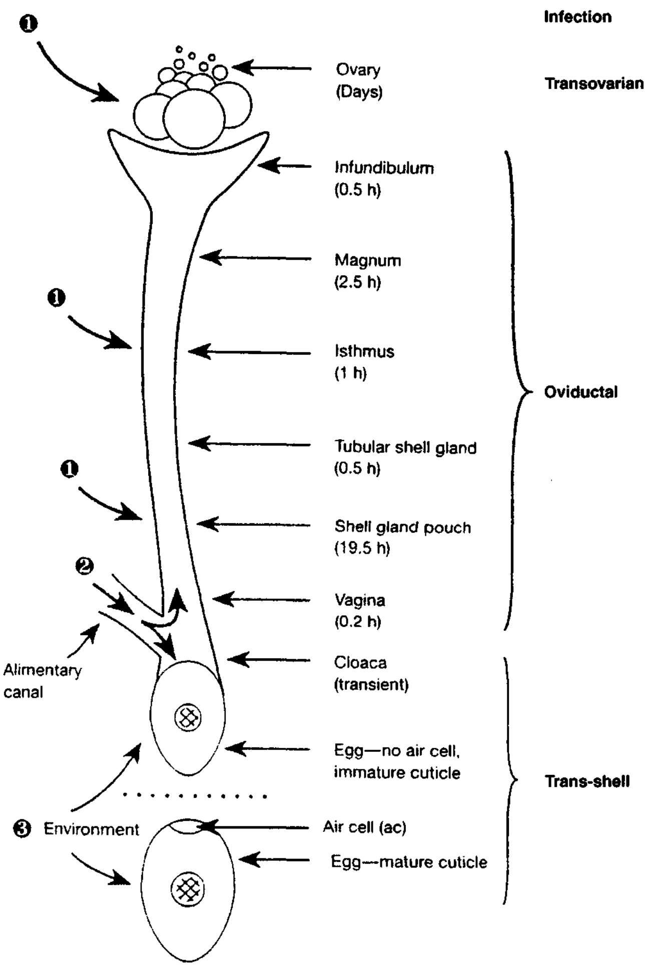

Figure 24-1 A Timetable for the Deposition of Major Components of a Hen Egg Together with an Indication of Sites/Times of Contamination. *Source:* Copyright © 1994, R.G. Board and R. Fuller.

At oviposition, the avian egg has the gross structure depicted in Figure 24-3. At this time the yolk, which has a density greater than that of the white, is

Table 24-1 The Biological Properties of the Oviductal Proteins Present in the Albumen of the Eggs of Domestic Hens

Protein	*% of Albumen*	*Biological Properties*
Avidin	0.05	Avid binding of biotin
Riboflavin binding protein	0.8	Possible that riboflavin is bound such that microorganisms are denied this vitamin
Ovotransferrin	12–13	Avid chelation of Fe^{3+} dissociation constant ($\bar{K}_D$~10^{-29}M)
Cystatin	0.05	Inhibits thiol proteases ficin and papain (possible antiviral role also)
Ovomucoid	11.0	Inhibits trypsin
Ovoinhibitor	0.1–1.5	Binds trypsin, chymotrypsin, and several types of bacterial and fungal proteases
Ovalbumin	54.0	Structure indentifies it with serine-protease inhibitors but biological activity yet to be determined
Ovomacroglobulin	0.5	Serine protease inhibitory activity
Ovomucin	1.5–3.5	Endows albumin with high viscosity—this attribute enhanced through complex formation with lysozyme (inhibits viral hemagglutination)
Lysozyme	3.5	A 1, 4-ß-N-acetylmuramidase that cleaves the glycosidic bond between the Cl of N-acetyl muramic acid and the C4 of N-acetyl-glucosamine in bacterial peptidoglycans; contributes to integrity of albuminous sac through electrostatic interaction with ovomucin
Ovoglobulin G2	4.0	?
Ovoglobulin G3	4.0	?
Ovoglycoprotein	0.5–1.0	?

Source: Data from E. Li-Chan and S. Nakai, Biochemical Basis for the Properties of Egg White, *Critical Reviews in Poultry Biology,* Vol. 2, pp. 21–58, and H.S. Tranter, Lysozyme, Ovotransferrin and Avidin, in *Natural Antimicrobial Systems and Food Preservation,* V. Dillon and R.G. Board, eds., pp. 67–97, © 1994, CAB International.

at the center of the egg, a situation that is mainly due to physical properties of the inner and outer thick albumen. The former envelopes the yolk and at opposing poles it is twisted into rope-like structures, the chalazae, that merge with the outer thick white. The slightly elastic chalazae allow limited rotation but impede lateral displacement of the yolk. The two thick albumens differ from the inner and outer thin white in having a higher content of ovomucin. This glycoprotein occurs in two forms, α-ovomucin and the insoluble (fibrous) ß-ovomucin.[104] The latter has the higher content of carbohydrate residues. An interaction between lysozyme and ovomucin probably contributes also to the fibrous nature of the thick albumen.

24.3 CHANGES IN THE STRUCTURE AND COMPOSITION OF EGGS WITH TIME

Figure 24-4 summarizes the gross changes occurring in the egg of the domestic hen from oviposition onwards. Many factors, including breed,[31,132] health,[76,122] age of bird, and especially storage temperatures,[93,108] influence the rate of change. The following features need to be stressed:

- Changes in the relative densities of the yolk and white, such that the tendency of the former to sink in the white of a newly laid egg is reversed in storage. This results from the diffusive loss of water from the albumen via the pores in the eggshell and the movement of water from the albumen (freezing point –0.42°C) to the yolk (–0.57°C) due to osmotic forces (difference in osmotic pressure, approximately 1.8 atmospheres). The progressive stretching of the vitelline membrane leads to a loss of elasticity and strength.[32]
- Breakdown of the ovomucin-lysozyme complex in the thick albumen[104] results in the yolk floating upwards until contact is made with the inner shell membrane. If an egg is stored broad

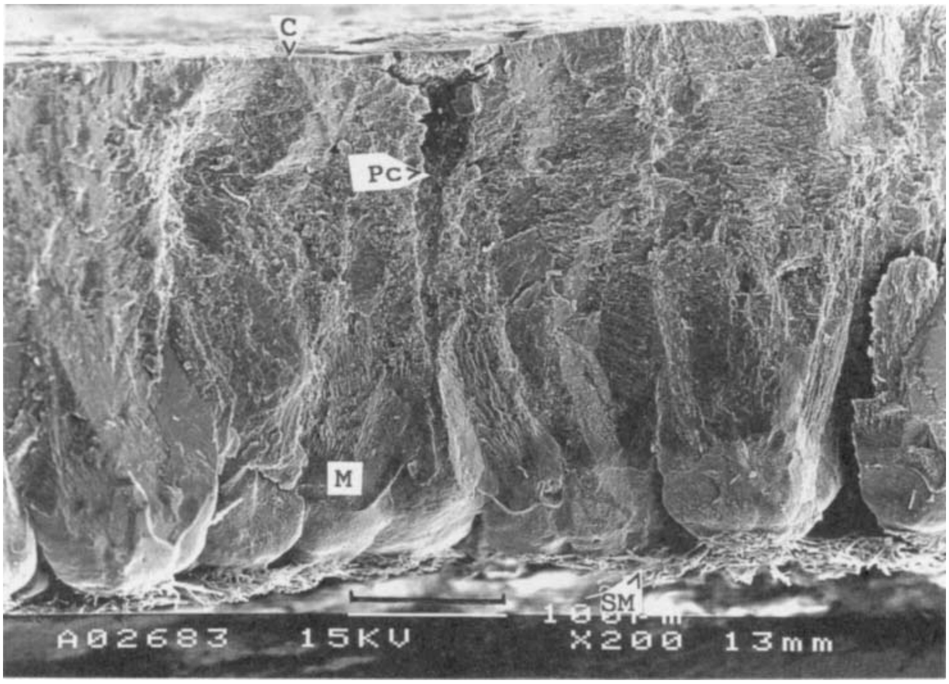

Figure 24-2 Radial Section of the Shell of a Hen Egg. PC, pore canal; C, cuticle; M, mammillary layer; and SM, shell membrane.

pole upwards, as is the case with the majority of eggs in commerce, the upward movement of the yolk will be arrested by the inner membrane of the air cell (Figure 24-4). This membrane moves progressively toward the center of an egg as the volume of the air cell increases to accommodate diffusive water loss.

24.4 THE ANTIMICROBIAL DEFENSE SYSTEMS IN EGGS

The antimicrobial defense systems of the hen's egg are listed in Table 24-2. The contributions of the first three components are well known. There is no direct evidence to implicate the fine structure of the yolk in the antimicrobial defense of an egg. The available evidence suggests that the yolk is devoid of any worthwhile defense apart from antibodies against organisms that have infected the hen.[106] Several workers, including Ayres and Taylor,[7] have demonstrated the rapid growth of bacteria inoculated into whole yolks with incubation at optimal temperatures for the test organism.[27] The mere act of inoculation may damage yolk structure such that bacterial growth is promoted. Burley and Vadehra[32] discussed three attributes of the yolk that could well have a bearing on this issue.

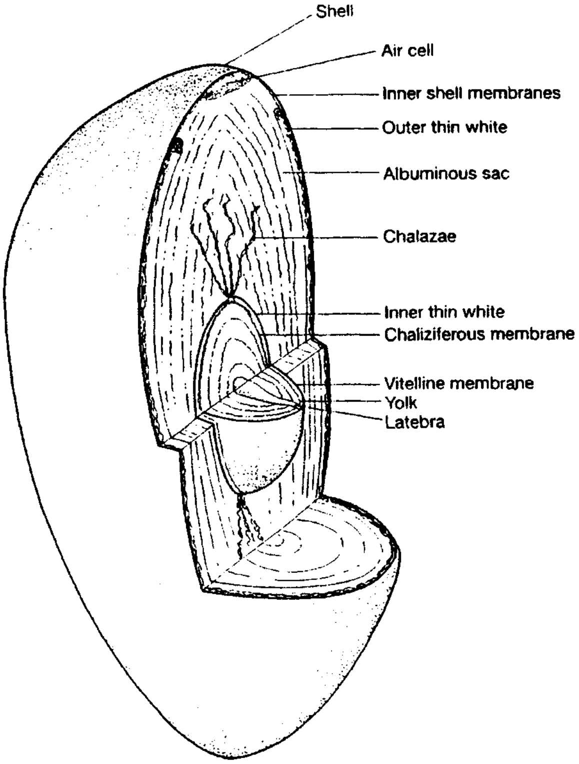

Figure 24-3 An Artist's Impression of the Hen Egg as Shown by Cut-away Sections. *Source:* Copyright © 1994, R.G. Board and R. Fuller.

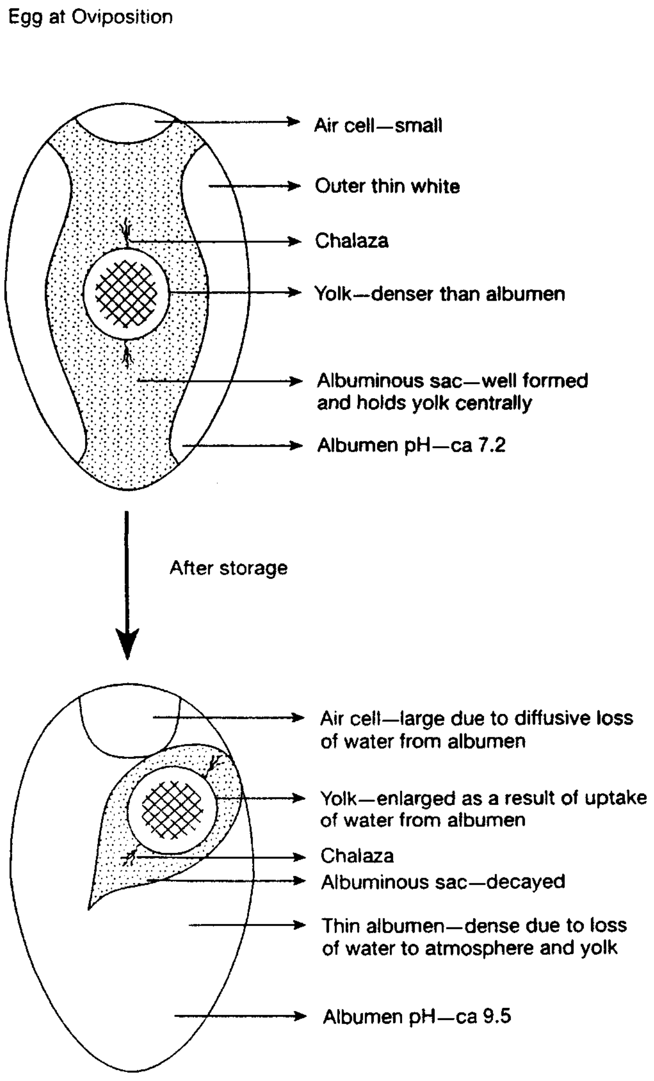
Egg at Oviposition
Air cell—small
Outer thin white
Chalaza
Yolk—denser than albumen
Albuminous sac—well formed and holds yolk centrally
Albumen pH—ca 7.2
After storage
Air cell—large due to diffusive loss of water from albumen
Yolk—enlarged as a result of uptake of water from albumen
Chalaza
Albuminous sac—decayed
Thin albumen—dense due to loss of water to atmosphere and yolk
Albumen pH—ca 9.5

Figure 24-4 A Summary of Changes Occurring in Eggs during Storage (reproduced with permission[21]). Many factors contribute to the changes occurring during storage *viz.* temperature, humidity of store, breed of hen, stage in laying cycle, health of hens, etc. Temperature is of paramount importance; slowest rate of change occurs in chilled eggs (*ca.* 4°C). *Source:* Copyright © 1994, R.G. Board and R. Fuller.

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1. The yolk consists of concentric layers of light and dark material. These do not fuse with time. Burley and Vadehra[32] concluded therefore that the yolk has an inherent stability that could well hinder diffusion of material between yolk and white.
2. Partitioning of the yolk with trilaminar membranes has been observed in fixed material examined with transmission electron microscopy. Such partitions are not present in yolk extruded through a hole in the vitelline membrane. Burley and Vadehra surmised that such partitioning impeded diffusion of material in the yolk.
3. Dolman and Board (unpublished) demonstrated a negligible increase in the glucose and amino acid contents of the albumen of eggs stored at 25°C for 42 days. There is also a marked and persistent asymmetry in the pH of the egg's contents.[105] At the time of lay the pH of the white is about 7.6 and that of the yolk 6.0. Within a short period of storage, the pH of the albumen rises to >9.5 as CO_2 is lost, whereas the yolk is still slightly acid after 100 days at 2°C.

All of the above need to be considered in any discussion of the integrated working of the antimicrobial defenses of eggs (Figure 24-4).

24.5 MICROBIAL INFECTION OF EGGS DURING PASSAGE THROUGH THE OVIDUCT

24.5.1 The Incidence and Extent of Microbial Contamination of Egg Contents

These have been contentious issues since a pioneering study in 1873. In that year Gayon,[56] with the support of Pasteur, refuted the claims of the protagonists of spontaneous generation, namely that vigorous shaking induced putrefaction of an egg. From a review of studies done over the next 50 years or so, Romanoff and Romanoff[105] noted that incidences of contamination of 3% to 87% of egg contents had been reported and that upwards of 40 "species" of bacteria had been isolated. They surmised, "it is quite likely that these figures were obtained because of the technical difficulties encountered in working with material of such unusual consistency and physical structure." Haines[61] was of the same opinion, "It may be stated categorically that it is impossible to make a completely satisfactory bacteriological analysis of an egg." These opinions are still valid, even allowing for the introduction of safety cabinets and improved aseptic techniques. Moreover, the identification of a possible infection site in the white is largely guesswork. Neither of these issues is of importance in the case of rotten eggs because the evidence supports but one conclusion, rot-producing microbes infect eggs post-oviposition (Figure 24-1). They are of importance with pathogens that cause vertical infection in poultry flocks or those that are passed via the egg contents to humans. Barrow[13] stressed that a number of virulence factors are re-

Table 24-2 The Antimicrobial Defense Systems of the Hen Egg

Component	Mode of Action
Biological structure	The compartmentalization of an egg's contents (Figure 24-3) ensures that at oviposition and for a short period thereafter the yolk—the most vulnerable entity in terms of microbial breakdown—is located at the greatest distance from microorganisms that infect the albumen via the trans-shell route
The cuticle, shell, and shell membranes	A physical barrier to trans-shell infection of the albumen (see Figure 24-2)
The white	**Physical** The gelatinous/fibrous nature of the white (a) impedes bacterial movement, and (b) acts as a shock absorber such that the vitelline membrane and the organization of the yolk are protected form physical damage
	Chemical The albumen is an inadequate medium for microbial growth because of the alkalinity (pH 9.6), its low content of simple nitrogenous compounds, and the presence of oviductal proteins having a range of biological properties (see Table 24-1)
The vitelline membrane	A physical barrier that impedes the ingress of microorganisms to the yolk
The yolk	Fine structure is the most likely impediment to the diffusion of small molecules into the surrounding albumen

quired for the survival of a microorganism in the hen's alimentary tract and its bloodborne passage to, and persistence in, the reproductive tract, with contamination of the egg contents as a possible sequel. The virulent, host-adapted avian pathogens, *Salmonella gallinarum* and *S. gallinarum-pullorum,* pass from hen to progeny by this route. With the latter, egg infection occurs most commonly after the egg has been released from the ovary. Barrow contends that the majority of eggs will be infected in the vitelline membrane or albumen. Ova in follicles may be infected but, if such were included in an egg, embryogenesis would be unlikely to take place and hence vertical transmission would not occur. The recent "pandemic" in humans associated with *Salmonella enteritidis*[5] has revealed that a non–host-adapted serotype of this genus, but one with virulence determinants that aid carriage by a systemic infection of domestic fowls,[54,58,73] does mimic *S. gallinarum-pullorum* in that it enters the oviduct, possibly via the vascular system in many cases, and infects the contents of the egg. The oviduct can become infected with salmonellas that move up from the cloaca. The available evidence suggests that, with some eggs infected with *S. enteritidis* by either route, embryogenesis proceeds normally and a healthy (even if infected) neonate is produced.

Methods of poultry husbandry can influence infection of the oviduct and the incidence, and perhaps the periodicity also, of egg contamination with salmonellas. Thus, Reiber et al.[103] isolated salmonellas from semen taken from cockbirds and there is evidence[101,102] that semen may serve as a vehicle for transmission of salmonellas to the hen, which may result in sporadic production of contaminated eggs. The incidence of salmonella in the crops[63] of laying hens increased significantly when hens were starved for 24 hours.[72] Hens nearing the end of their laying cycle are sometimes deliberately stressed (by starvation, no access to water, or reduction in lighting) in order to induce a molt, because egg production returns to a commercially acceptable level following a molt. Starvation has been associated with an increased incidence of both systemic infection in, and fecal shedding of, salmonellas by laying hens.[67–69] In an outbreak of salmonellosis among the customers of a restaurant, eggs containing *S. enteritidis* were implicated and their source identified.[120] It is of interest to note two features of the implicated production farm:

1. Some hens were shown to be carriers of *S. enteritidis* a few weeks before the outbreak.
2. A few weeks before the outbreak strife between management and labor caused the hens to be deprived of water and food for 2 to 3 days.

In view of these examples of the probable influence of stress on the interaction between the hen and salmonellas, it is tempting to speculate that the caged hens studied by Humphrey and his collaborators[74] may have been unwittingly stressed on some occasions. This could have been the reason for the observation that the incidence of infected eggs tended to be clustered in just a few days of what was a protracted study that sought to establish the incidence of salmonella contamination in eggs laid by naturally infected hens.

The results obtained from surveys with this overall objective must be considered in the context of (1) the history and management of the flock(s) producing the eggs included in a survey, and (2) the bacteriological methods used in a survey. It is evident from the above discussion that stress is likely to lead to a sporadic increase in the incidence of egg contamination above background levels. As enrichment culture is essential in order to increase the number of salmonellas in freshly laid eggs to levels that can be detected and isolated by direct plating, the choice of sample size of white or whole egg contents and enrichment methods will be important in the case of (2) above. This was exemplified in a recent study[53] of pooled egg contents spiked with *S. enteritidis* 13a. Thus, direct plating on brilliant green agar from a pooled sample immediately after spiking required an inoculum 10^4-fold larger than in a sample that had been incubated with an enrichment medium for 24 hours before plating. Moreover, supplementing samples of the pooled contents of 10 eggs with Fe increased significantly ($P<0.005$) the percentage of pools (inoculum size, fewer than 10 salmonella per pool) that were demonstrated by direct plating to be infected.[55]

As noted above, it is difficult to sample viscous albumen aseptically; thus, caution must be exercised when assessing the results of surveys that sought to establish the incidence of *Salmonella* contamination of egg contents. In a review of data obtained from an investigation of the contents of 8,698 eggs obtained from 22 flocks (commercial layers both battery and free range, layer and broiler breeds, and experimental), Humphrey[71] recorded a range in the incidence of contamination with *S. enteriditis* of from 0.1% to 1.0% (average, 0.6%) of eggs. He noted that only a few salmonella occurred per egg and deduced that the outside of the vitelline membrane or the albumen surrounding it were the principal sites of contamination.

From a review of the literature on *Salmonella* infection of eggs,[45] it was noted not surprisingly that widely differing frequencies of internal infection in eggs have been reported *viz.* 5 out of 10 eggs from a small domestic flock, 10 of 613 eggs from a deliberately infected flock of 42 hens, and 11 of 1,119 eggs from two naturally infected flocks of 23 and 15 birds. Some infected eggs contained less than 10 *S. enteriditis* PT4 per egg. Vugia et al.,[143] who investigated an outbreak of salmonellosis among customers of a chain of restaurants, deduced that 1 in 12 of the eggs from the farm supplying the restaurant may have been infected with *S. enteriditis* at the time of the outbreak. In summary, the available evidence reveals that eggs are infected in the oviduct with *S. enteriditis.* It does not allow, however, conclusions about the incidence or the precise site of infection in eggs.

24.5.2 Infection in the Oviduct

As one end of the oviduct is located in the cloaca, there is at least a theoretical possibility of microorganisms migrating up the lumen of this organ. This possibility was examined by several workers in the early years of the 20th century. From a review of their observations, it was concluded[61] "that the healthy avian oviduct approximates to sterility and has, in fact, mechanisms tending to rid it of occasional invading microorganisms." Phagocytosis, mechanical action of the oviduct, which tends to force contents of the lumen toward the cloaca, and the bactericidal action of unidentified secretions have been cited[29] as possible agencies involved in the removal of causal contaminants from the oviduct. The possible susceptibility of the oviduct to ascending infection has been largely ignored since the review by Haines.[61] As noted by Bruce and Drysdale,[29] the few studies that have been done dealt with very few birds. They collected 656 fertile eggs over a 10-day period from 140 individually caged birds (broiler breeders) that were coming to the end of their laying cycle. The eggs were incubated, and the incidence and type of contamination of eggs that failed to hatch assessed; fewer than 4% of such eggs were contaminated. The hens were slaughtered, both ends of the oviduct were clamped before removal from the carcass. The lumen of the oviduct was rinsed with 10 ml of Ringers solution, and the number and types of bacteria in this suspension determined. Sixty-three percent of the oviducts were contaminated, the level of contamination ranging from $\leq 10^3$ to $>10^6$ organisms/oviduct. The following organisms were isolated: *Micrococcus* spp., *Bacillus* spp., *Lactobacillus* spp., *Escherichia coli,* nonfermentative Gram-negative rods, and nonsporing Gram-positive rods. A different range of organisms was isolated from eggs that failed to hatch. A similarly diverse range of organisms was recorded by Harry[64] who studied the contamination of the oviduct and ovaries of hens. It is notable that the diversity recorded by these workers matched that of earlier ones (reviewed by Romanoff and Romanoff[105]) who characterized the contaminants of egg contents. Indeed such diversity poses important questions. Is it possible to adopt appropriate and rigorous aseptic procedures such that there can be absolute certainty that isolates from egg contents or the lumen of an oviduct were truly indigenous to the sample being examined? If this question is answered in the affirmative, then another question must be posed. Are the chances of contamination of eggs in the oviduct so high that oviductal proteins have been selected during evolution to protect the yolk from chance contamination in the oviduct as well as from colonization by saprophytic microorganisms post-oviposition? This topic is discussed further in Section 24.7.

24.6 THE MICROBIOLOGY OF ROTTEN EGGS

The microbiology of rotten eggs attracted a great deal of attention in the first half of the 20th century. The reason for such interest was noted by Maurer, "He who has seen the wagon loads of rotten eggs hauled from the establishments of our big egg dealers fully appreciates the tremendous waste connected with the industry."[86] When Maurer made this observation about conditions in the US, egg production was on a small scale and little attention was given to the need to collect eggs regularly, clutches of eggs were taken occasionally from the nests of hens that chose to lay away from the poultry house and, in the absence of controlled lighting, eggs produced during the spring/summer flush were stored for long periods. Such conditions still prevail in some parts of the world. With storage, diffusive water loss from eggs results in weight loss and a reduction in value. Consequently, particular attention was directed at the selection of a humidity that minimized weight loss and mold growth. Fragile white growth of mold on the shell surface was sometimes taken as evidence that the humidity of the store was being maintained at a satisfactory level.[115] If, however, the relative humidity was maintained at 96% to 100% for three months at chill temperature, then dark mold growth developed on the shell and the egg contents became infected with hyphae that had penetrated the pore canals. Species of *Penicillium, Sporotrichum, Thamnidium, Mucor,* and *Alternaria* were isolated from eggs stored under very humid conditions.[61,144] Sharp and Stewart[115] suggested that failure of molds to grow at relative humidities less than 94% may have been associated with "a restriction of the food supply as controlled by moisture." Two strains of protease-producing (cuticle-digesting) pseudomonads were isolated from the surface of shells of eggs stored in very humid conditions.[23] Yeasts also grew on the shell surface, but their growth appeared to be dependent upon nutrients released from the cuticle by the pseudomonads. High incidences of mold growth and spoilage of eggs are, of course, a thing of the past in developed countries with their intensive poultry industry. It may well not be so in countries in which small egg producers are still major suppliers to unsophisticated markets. Even so, the occasional rotten egg does reach the consumer in developed countries, and the consumer's reaction to the smell and appearance of a rot may well influence future purchases.

Gram-negative bacteria are the dominant cause of rots in stored eggs.[30] In studies conducted between 1938–1947 on addled eggs derived from three states in Australia,[1] a total of 3,873 rotten eggs were examined. Of these 1,475 were identified as green rots (causative organism, *Pseudomonas fluorescens*), 1,079 as colorless rots (*Pseudomonas, "Achromobacter"* spp.), 1,142 as black rots (mainly *Proteus* spp. but occasionally *Pseudomonas* spp.), and 121 as pink rots (strains of *Pseudomonas*). This study revealed also the importance of storage temperature and farm. No rots were evident, as demonstrated by candling, in eggs stored for 6 to 8 weeks at 0° to 0.6°C (the temperature of the storage compartment on ships used to transport eggs from Australia to the United Kingdom [UK] at that time). With subsequent storage at ambient temperature for 24 to 26 days, 19.1% (range 1–65%) of the eggs developed rots. A high incidence of rots was associated with eggs coming from farms that machine-cleaned eggs.

An extensive study was done in 1950 of rots in eggs obtained from farms and packing stations in British Columbia, Canada.[136–138] With 16,652 eggs taken directly from farms and stored for 6 to 7 weeks at 16°C, an overall spoilage rate of 3.19% was found. None of the eggs from some farms spoiled, whereas a 26% spoilage rate was noted with those from one farm. An insignificant incidence (0.42%) of spoilage occurred in clean eggs collected from nest boxes containing clean litter or from the wire floor of "roll away" nests (a system common in battery cages). The well-being of eggs was unaffected by storage in dirty egg boxes. The incidence of rots in heavily soiled eggs from various farms ranged from 0.6% to 11.00% (average, 2.73%). The highest incidence of rots was found in eggs washed on the farm, varying from 0% to 19.8% (average, 7.44%) in one survey and from 0% to 22.6% (average, 6.06%) in another. Two hundred twenty-eight bacteria were isolated from 81 rots that developed in 591 eggs during 6 weeks storage at 25°C. Although these isolates were characterized in some detail,[50] the identity of species and, in one instance genus (for example, *Aeromonas* identified with *Proteus*), would now be considered dubious simply because of the inadequate identification schemes then available; even so, members of all the genera of bacteria that Haines[60] and Alford et al.[1] had associated with the addling of eggs were isolated.

These same genera were represented in the 345 bacteria isolated some 20 years later[17] from eggs that had rotted on the farm or under controlled conditions in the laboratory in the UK (Table 24-3). The main features of rots produced by particular bacteria and physiological attributes associated with such changes are given in Table 24-4. Microorganisms lacking all these attributes (eg, *S. enteriditis, Alcaligenes faecalis*) form large populations in eggs infected naturally or deliberately but cause only slight turbidity in the albumen. Eggs infected with these organisms would not be detected in routine candling nor by a cursory examination when broken out in a container that prevented light transmission through the albumen. This situation may have resulted in the use of eggs heavily contaminated with salmonella in the kitchen of the home or canteen, or inclusion in products in egg-breaking plants. Eggs containing large numbers of *"Achromobacters"* have been found among those awaiting processing in such a plant.[77]

It is common for addled eggs to contain two or more species of bacteria. If eggs are inoculated with pure cultures of these isolates, in general only one produces the changes noted in the original. The vast majority of spoilage bacteria are commonly present in soil, in water, in the deep litter on the floor of poultry houses, in decaying feces of hens, and on hay and straw used to line nest boxes.[65] Thus, infection of eggs after laying is of infinitely greater importance than oviductal infection. The genera of bacteria listed in Table 24-3 have been associated with the addling of eggs produced in Australia, South Africa, Canada, and the UK. There appears to have been no change in their incidence over a 30-year period. Thus, properties of eggs, rather than climatic conditions or farming practices, select rot-producing bacteria from what may well be a very heterogenous, initial infection. Gram-positive bacteria, commonly present in the oviduct,[29] are very rare contaminants of eggs that have rotted during storage at ambient or chill temperatures. These bacteria can be important contaminants of the contents of hen eggs incubated at 37° to 39°C. Seviour et al.[113] examined 1,160 "incubator clears," ie, eggs in which germinal development had not occurred and which were not opaque to transmitted light when candled. Twenty-five percent of the eggs contained large numbers of bacteria. When broken out, 13% of these eggs had cloudy whites and most of these had custard-like material on the surface of the yolk. Of the 350 bacteria isolated in pure cul-

Table 24-3 The Identity of Bacteria Isolated from Rotten Eggs[a]

Identity	*Occurrence in Eggs*	*Type of Rot Produced in Inoculated Eggs*[b]
Pseudomonas		
fluorescens	Common	Pink rot
putida	Common	Fluorescent green rot
aeruginosa	Uncommon	Fluorescent blue rot
Stenotrophomonas maltophilia	Uncommon	Green rot
Alcaligenes faecalis	Common	No change
Flavobacterium spp.	Very uncommon	Yellow pigment in shell membrane
Cytophaga spp.	Very uncommon	Yellow pigment in shell membrane
Aeromonas liquefaciens	Uncommon	Black rot type 1
Proteus vulgaris	Common	Black rot type 2
Cloaca spp.	Common	Custard rot or no change
Serratia marcescens[c]	Uncommon	Red rot
Citrobacter spp.	Common	No change
Bacillus, Arthrobacter, Micrococcus, and *Streptococcus* species isolated occasionally.		

[a] Based on a study of 345 isolates from eggs that had rotted at the poultry farms or under controlled conditions in the laboratory.

[b] Description of rots based on Haines[61] and Florian and Trussell.[50] See Table 24-4 for additional details.

[c] An uncommon rot in hen eggs described by Zagaevsky and Lutikova[146] and in duck eggs by Panda and Rao.[99]

Table 24-4 Characteristic Features of Rots Produced in Hen Eggs by Bacteria and Key Attributes of Causative Organisms

Rot (causative organism)	*Changes in Egg*	*Pig.*[a]	*Prot.*[b]	*Lec.*[c]	*H_2S*
Pink rot *(Pseudomonas fluorescens)*	Mealy yolk; fluorescent green albumen	+	+	+	–
Fluorescent green rot *(Pseudomonas putida)*	Fluorescent green albumen	+	–	–	–
Fluorescent blue rot *(Pseudomonas aeruginosa)*	Fluorescent blue albumen	+	+	+	–
(Stenotrophomonas maltophilia)	Gelatinous, amber-like yolk striped with olive green pigment, strong almond smell	–	+	–	+
Black rot, type 1 *(Aeromonas liquefaciens)*	Gray, watery albumen; gelatinous yolk; blackened throughout	–	+	+	+
Black rot, type 2 *(Proteus vulgaris)*	Dark brown albumen; dark brown, mealy yolk	–	+	–	+
Custard rot *(Cloaca* spp.)	Yolk encrusted with custard-like material	–	–	+	–

[a] Pig., water soluble pigment produced.
[b] Prot., proteolysis.
[c] Lec., lecithinase production.

ture, 74 were identified with *Staphylococcus,* 205 with *Micrococcus,* and 36 with coliforms (25 as *Escherichia coli).* The origin of these contaminants is unknown, but the production of eggs in a dusty atmosphere may have led to contamination of the shell membranes. Formaldehyde fumigation of the eggs may have played a part also. *Streptococcus* spp., *Micrococcus* spp., and Enterobacteriaceae were the principal contaminants found in heavily infected eggs among 2,542 incubator rejects.[113] A study of 95 addled eggs of domestic ducks obtained from 2 commercial hatcheries and >1,400 addled eggs of about 50 species of waterfowl from the hatcheries of the Wildfowl Trust, Slimbridge, Glos, UK, resulted in the isolation of 317 pure cultures.[113] Sixty-four percent of these were identified with *E. coli,* 8.5% with *Staphylococcus,* and 15% with *Micrococcus* spp. All of the *E. coli* isolates from waterfowl eggs formed large mucoidal colonies (individual cells encapsulated) when grown on media containing glucose or lactose. None of the *E. coli* from hen eggs did so. *Escherichia coli* was an important cause of colorless rot in duck eggs obtained from various markets in Mysore.[99] Differences in the biological properties of the oviductal proteins of ducks and hens, rather than differences in husbandry practices, may be responsible for the selection of such different microbial associations in the eggs of the two groups of birds. The poor public health record of duck eggs in the context of salmonellosis in humans[110] may be linked to the selection of "smooth" rather than "rough" strains of Enterobacteriaceae by the duck egg white rather than the "porosity" of the shell, a commonly cited reason.[145]

24.7 COURSE OF MICROBIAL INFECTION OF EGGS

There are three possible routes for the initial infection of an egg's contents (Figure 24-1). (1) **Transovarian infection**—the yolk within the follicle of an ovary is infected with bloodborne microorganisms. This route is of particular importance in hens infected with host-specific pathogens.[13] It will not be discussed further because it appears unimportant in the context of table eggs or egg products. (2) **Oviductal infection**—the vitelline membrane or egg white is infected during transit along the oviduct. Microorganisms are introduced into the lumen of this organ via (a) the vascular system, (b) upward migration from the cloaca,[5] or (c) husbandry practices such as artificial insemination.[103] Contamination of the oviduct is accentuated in stressed hens.[67] (3) **Trans-shell infection**—microorganisms on the surface of the shell are translocated to the underlying shell membrane.[18] This may be an active process. If eggs are stored in very humid conditions, molds grow on the shell surface and hyphae penetrate the pores in the shell. Infection passes inwards from the shell membranes until eventually the entire white and yolk are colonized.[23] This means of infection is extremely uncommon in eggs produced by intensively managed hens and sold through controlled marketing channels. Bacteria have a passive role in trans-shell infection under normal commercial conditions. Practices such as egg washing may cause the translocation of bacteria from the outside of the shell to the underlying shell membranes.[29]

There is a common sequence of events in the progression from the initial (light and localized) to the systemic and heavy infection of the entire contents of eggs infected by the oviductal or trans-shell route. There is a lag period of very variable duration followed by a short phase of rapid bacterial multiplication. Subsequently, the white or, more commonly, both the yolk and white will become grossly changed in appearance (Table 24-4).

The progression to a generalized infection of an egg's contents can be interpreted when the course of infection is considered in the context of the workings of a complex antimicrobial defense system.

24.7.1 Trans-Shell Infection

The cuticle-pore complex, shell, and shell membranes (Figure 24-2) are potential barriers to trans-shell infection of egg contents.[18,90] Romanoff and Romanoff[105] were probably the first investigators to identify the cuticle's contribution to the egg's defense. Sparks and Board[124] demonstrated that the barrier properties of a mature cuticle are much greater than those of an immature one. Thus, there was 100% infection of the contents of eggs placed on fresh hen feces when the cuticle on the shell was wet and lustrous but only a 16% infection rate when it had dried out. Infection of the contents occurred in all 18 freshly laid eggs sprayed with *Salmonella typhimurium*.[95] When freshly laid eggs were partially submerged in relatively dry nest litter contaminated deliberately with this organism, the contents of 10 out of 17 eggs were contaminated.[95] The incidence of contamination of eggs of both layer and broiler strains of hens laid in the litter on the floor of the poultry house was invariably higher than of those laid in nest boxes.[119] These observations indicate that contamination of egg contents is highly probable if an egg is laid in a contaminated environment. This situation is recognized by the management of poultry breeding farms. Egg collection and nest hygiene have been identified as two of the "key critical control points" in an integrated system of *Salmonella* control of hatching eggs. The following procedures are commonly in place: (1) collection of eggs from nests four times per day, (2) cleaning of nests and sanitizing, eg, with formaldehyde prills once every 14 days, and (3) monitoring standards of egg hygiene by laboratory tests on a monthly basis.

Until recently there was a concensus view that penetration of contaminated water into the pore canal (Figure 24-2) was an essential step in trans-shell infection of eggs having mature cuticles.[62] Penetration rarely if ever occurs simply through contact of a shell with water. The microstructure of the surface of the cuticle, especially that adjacent to the shell in the outer pore orifice, is such that the contact angle (Θ) required for capillary attraction of water rarely occurs. It does so on occasions in eggs from which the cuticle has been removed with EDTA.[123] Work is generally needed to force water into the pore canal. This can be done in three ways.[22]

1. When a warm egg is immersed in cold water, water is pulled into the pore canals as a result of a partial (negative) pressure generated by the reduction in volume of the yolk and white being greater than that of the space enclosed by the shell.
2. Eggs immersed in water are exposed to a pressure less than atmospheric, and then normal pressure is suddenly reimposed.
3. Eggs immersed in water are subjected to a pressure greater than atmospheric.

The shell of the hen's egg contains $1.0 - 1.7 \times 10^3$ pores. Porosity was a nebulous term until the work of Ar et al.[6] They defined porosity thus:

$$G_{H_2O} = \dot{M}_{H_2O} / \Delta P_{H_2O}$$

where G_{H_2O} = water conductance expressed as mg of water loss per mm Hg of difference in water vapor pressure across the shell per day

$\dot{M}_{H_2O}$ = water loss from the egg (mg d^{-1})

ΔP_{H_2O} = difference in water vapor pressure across the egg shell (mm Hg)

G_{H_2O} is a measure of the number and size of the pores. It is constant once shell formation is complete.

No correlation was found[123] between the weight of water taken up by a warm egg (37°C) immersed in iced water and the porosity of its shell as measured by G_{H_2O} or the number of pores per shell ($r = 0.03$; P 0.05; $n = 70$). The amount of water taken up by eggs with shells from which the cuticle had been removed with EDTA was more than that of the control eggs but again there was no correlation between water uptake by, and water vapor conductance of, a cuticleless egg. No correlation was evident when the amount of water taken up by an egg was regressed against the amount of cuticle (dry weight) cm^{-2} of shell ($r = 0.50$; $P<0.05$; $n = 48$). Fissures resembling cracks in dried mud are a feature of the outer surface of the cuticle (Figure 24-2), especially that covering the outer orifice of a pore. Sparks and Board[123] measured the length, but not the width, of the fissures in the cuticles of groups of five eggs selected from those that had been shown to take up high, intermediate, or low amounts of water. Fissure length regressed against water uptake gave a good correlation ($r = 0.9$; $P<0.05$). No such correlation was evident when fissure length and water uptake for every one of 24 eggs were analyzed. The length and extent of branching of fissures in the cuticles of eggs cooled rapidly (forced convection with an air speed of 30.5 M min^{-1} and a temperature of 0°C) was greater than on eggs cooled by ambient storage.[49] One hundred percent of the first group of eggs but only 48% of the second were infected by the trans-shell route with *S. enteritidis*. In practice, there is only a very small weight change when a warm egg is immersed in iced water; 3–415 mg for normal eggs and approximately 350–610 mg for cuticleless ones.[22,123] The addition of a surfactant, Triton X-100, to iced water increased the weight gain of eggs.[123]

The shell membranes behave as semi-permeable membranes when they separate a fluid of high from one of low molarity. There is unhindered water uptake when a shell-less egg or one from which a small piece of shell has been removed is immersed in water. The wall of a well-formed pore canal (Figure 24-2) obviously imposes a drag sufficient to negate osmotic forces operating across the shell membranes in eggs. Work is therefore needed to flood a normal pore canal and to ensure also that water reaches the underlying membranes; the magnitude of the drag is not apparently changed by repeated flooding of the pore canal. Mean weight gains of 72.0 ± 13.0 (SD) mg of eggs ($n = 60$) subjected to method 3 above on one occasion and 130.0 ± 21.0 mg ($n = 60$) on two occasions have been reported.[96]

Various methods, including the addition of a pigment (eg, blue lake[77]), carbon black, and/or microorganisms to the slush ice,[22] have shown that flooding of the pore canals results also in particles being lodged in the underlying shell membranes. The number of pores flooded when eggs are immersed in slush ice varies. When a green pigment and carbon black were added to slush ice used to chill 97 white eggs, 48% had 0 to 19 green spots with black centers on the membranes, 35% had 20 to 99, and 17% had >100. With 195 brown eggs, approximately 80% had 0 to 19 spots on the shell membrane, 15% had 20 to 99, and 5% had >100.[22] About 80% of the brown eggs having from >80 to 100 black-centered spots on the shell membranes had no cuticle on the entire shell, cuticle on one pole only of the shell, or a grazed cuticle. The last fault was caused presumably when an egg with an immature cuticle was forced over the wire floor of a battery cage. Eggs of all three categories occurred (but at a very low incidence) in the 80% of brown eggs that had 0 to 19 spots per shell membrane. The outer orifices of some pores are evidently plugged even though a stain fails to demonstrate cuticle on the surface of the shell. With other eggs, large numbers of colored spots occur on the shell membrane even though the shell surface is deeply and evenly stained. Cuticle presumably bridges, rather than plugs, the outer orifice of a pore, the latter being flooded when the bridge collapses under pressure. The effectiveness of the cuticle can be undermined by the following:

1. Innate properties of hens. These can affect the quality of the cuticle on their eggs on a day-to-day basis[10] as well as a hen-to-hen basis.[24]
2. The quality of the shell upon which the cuticle is laid down. Thus, carbon black penetration of white eggs is commonly associated with calcareous lumps on the shell.[22] Alls et al.[2] noted that antibiotic penetration was most pronounced through "sandy" areas of a shell or in the region of lumps on the shell surface. Such lumps, which can be persistent features on eggs laid by particular hens, cause malformations in the pores in the adjacent shell.[123]
3. The conditions to which an egg is exposed before bacterial challenge. Thus, attrition between shell surfaces and nest floor or brushes in washing machines,[121] forced cooling,[42,49] choice of chemicals for egg washing,[78] and microfractures in the shell[28] all compromise the effectiveness of the cuticle.

The above discussion was based on a concensus view, namely that the cuticle-pore complex is the major barrier to trans-shell infection. This view, which had its genesis in the work of Haines and Moran,[62] has been challenged by Solomons and coworkers.[89,91,118] They claim that the translocation is influenced mainly by shell quality and the microstructure of the mammillary layer (Figure 24-2). The specific gravity of an egg (determined by immersing an egg in a series of sodium chloride solutions of increasing concentration and identifying the one that allows the egg to float) is closely correlated with shell quality, especially shell thickness.[126] Bacterial penetration of the shell of eggs having a specific gravity (SG) of 1.07 was greater than that of those with an SG of 1.08, and penetration of the latter was greater than in those with an SG of 1.09.[94] In practice these results may well reflect in part the influence of the drag factor of the pore canal in trans-shell infection; the thicker the shell (*viz.* eggs of SG 1.09) the greater the drag factor of the pore canal and, hence, the lower the probability of bacteria being translocated through the pore to the shell membrane.

Solomons and coworkers[121] found no correlation between bacterial penetration of the shell and pore numbers or quality of cuticle but a good correlation with various faults in the structure of the mammillary layer. It is not clear from these studies whether or not such faults caused malformation of pore canals arising at, or adjacent to, the malformed cones in the mammillary layer.

Eggshells are contaminated by contact with nesting materials, the hands of poultry workers, and surfaces in the packing/grading room.[19,107] The range in the level of contamination of eggs from one source can be very large *viz.* $3.0 \times 10^2 - 1.0 \times 10^7$ for clean grade A eggs taken from a packing station (Table 24-5). Gram-positive bacteria are commonly the dominant contaminants of egg shells (Table 24-6) whereas Gram-negative ones are the principal cause of rots in eggs (Table 24-3).

Although it is evident that we still have a poor understanding of the physics of water and particle transfer along pores in the eggshell, the available evidence has allowed effective egg-washing practices to be devised.[30] Implementation of the following measures[8] allows washing of table eggs with a minimum level of spoilage during storage. Likewise these measures ought to allow also effective sanitization of hatching eggs on the production farm, a practice that is intended to replace formaldehyde treatment of eggs at a hatchery:

1. Wash eggs soon after lay.
2. Ensure that the temperature of water used for washing and rinsing is at least 10°C above that of the eggs.
3. Use an approved detergent-sanitizer.
4. Include a sanitizer in the rinse water.
5. Routinely check the concentration of detergent-sanitizer in wash and rinse water.
6. Clean egg-washing machines daily.
7. Ensure that wash water has a low content of Fe^{+++} (aeration of water entering a distribution system ensures a low level contamination with

Table 24-5 Levels of Bacterial Contamination of the Shell of the Hen Egg

		Number of Microorganisms		
Source of Eggs	*No. of Eggs Examined*	*Mean*	*(Range)*	*Ref.*
Farm (handled with gloved hands)	36	9.5×10^3	(NG[a])	107
Farm (normal handling)	36	1.5×10^5	(NG)	107
Packing station	72	7.0×10^4	(NG)	19
Battery hens	25	2.2×10^4	(2.5×10^3–8.1×10^4)	19
Hens on deep litter	25	3.5×10^5	(6.2×10^3–2.4×10^6)	19
Shops and farms	96	3.1×10^6	(5.0×10^2–1.0×10^7)	
	130	1.3×10^5	(NG)	107
Packing station				
Clean Grade A	NG	2.2×10^5	(3.0×10^2–1.0×10^7)	19
Lightly soiled Grade B	77	9.7×10^5	(1.0×10^3–1.9×10^7)	19

[a]NG = Not given.

Table 24-6 The Microflora of the Shell of the Hen Egg

Organism	Eggs from Shops and Farms	Eggs from Packing Stations: Clean	Eggs from Packing Stations: Lightly Soiled	Eggs from Packing Stations: Cracked
Staphylococcus	5[a]	9	5	11
Micrococcus	18	37	52	42
Bacillus	30	–	2.5	–
Pseudomonas/ "*Achromobacter*"	25	24	14.5	25
Coli-aerogenes	5	10.5	7.5	4
No. organisms examined	100	130	164	126

[a]Percentage of isolates; — none detected.

Source: Data from R.G. Board et al., The Microbiological Contamination of Egg Shells and Egg Packaging Material, *Poultry Science*, Vol. 43, pp. 584–595, © 1964, Poultry Science Association, Inc.

the element; water taken directly from a bore hole and used without aeration may contain relatively high levels of Fe^{+++}).

8. Place washed and dried eggs in a chiller as soon as possible after washing.

24.7.2 The Lag Period

There is a lag period of 7 to >20 days between trans-shell infection of freshly laid eggs and gross contamination of their contents (Figure 24-5) during storage at ambient temperatures.[21] There is a lag also before the growth of salmonellas in eggs infected by the oviducal route.[75] There is no such period in eggs infected by the former route when bacteria are suspended in water containing >2 ppm Fe^{+++}.[15] The specific gravity (SG) of eggs influenced the duration of the lag period. Eggs with SG >1.082 showed signs of rot 8 days (on average)

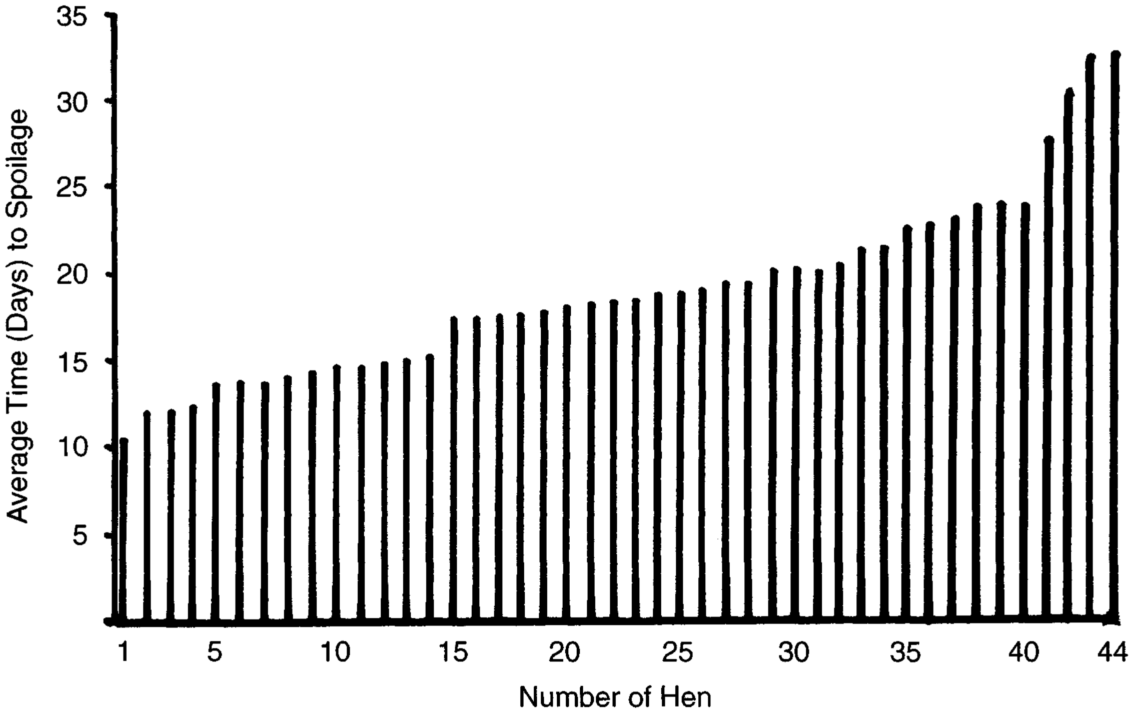

Figure 24-5 *Pseudomonas* Spoilage of Eggs Laid by Individual Hens. Eggs from Leghorn hens stored at 30°C for 24 hours. A chilled (5°C) culture of a fluorescent pseudomonad was poured over the warm eggs. After 10 minutes, the eggs were removed from the culture, dried and stored at 20°–21°C in a store having a relative humidity of 70% to 90%. On every third day during storage, the incidence of bacterial spoilage was established by candling with a UV lamp. Three or four eggs laid consecutively by individual hens were included in the experiment. *Source:* Adapted with permission from V. Orel, The Pseudomonas Spoilage of Eggs Laid by Individual Hens, *Poultry Science*, Vol. 36, pp. 8–12, © 1959, Poultry Science Association, Inc.

later than those having a lower SG.[94] The extent of bacterial penetration of the shell was greater in eggs of SG 1.07 than in those of SG 1.08. The shell's contribution to the defense appears to be determined also by the actual site of bacterial penetration (Figure 24-6). Thus, a far higher incidence of spoilage occurred in eggs challenged with bacteria after the pointed pole had been waxed *vis-à-vis* those whose broad poles had been waxed.[142] This must be considered in the context of the direction of movement of the yolk relative to the site of infection (see below).

It is obvious from Figure 24-7 that the albumen plays a central role in the antimicrobial defense of eggs. The duration of its contribution varied markedly (from 8 to 28 days; Figure 24-7) and is determined in part by the size of the microbial challenge. On average the lag was 9.9 days in eggs inoculated in the shell membrane with 6.0×10^5 pseudomonads but 22 days with an inoculum of 6.0×10^2.[27] There is also a marked variation in the duration of the albumen's defense when whole eggs broken out in plastic pots are inoculated with a piece of shell membrane infected with *S. enteritidis*.[83]

When various serotypes of *Salmonella* were inoculated into albumen (pH 9.2) alone, the majority failed to increase in numbers during 42 days' incubation at 20° or 30°C.[84] A few serotypes grew sluggishly throughout incubation. The addition of sufficient Fe^{+++} to saturate ovotransferrin in albumen induced rapid multiplication of all the serotypes, even those that had remained quiescent throughout 42 days' incubation. Large populations were formed within 24 hours at 25°C in albumen supplemented with ferric ammonium citrate alone or Fe^{+++} in combination with ammonium citrate. The addition of ammonium citrate and a range of growth factors had the same effect, but neither of these alone induced the growth of salmonellas. When depletion of glucose from albumen was used as an index of the effect of supplements,[34,35] the addition of Fe^{+++} and a source of combined nitrogen were essential for the rapid and complete removal of this substrate.

A lag period was a feature also in eggs inoculated with washings from dirty egg shells[112] or mixed cultures of bacteria isolated from eggs.[44] Gram-negative bacteria attained numerical dominance *vis-à-vis* Gram-positive ones in the shell membranes during this lag period. When rapid growth led to a generalized infection of the yolk and white, *Pseudomonas* spp. (probably because of their simple growth requirements) became dominant in eggs stored at ambient temperature or below, coliforms at 37°C.

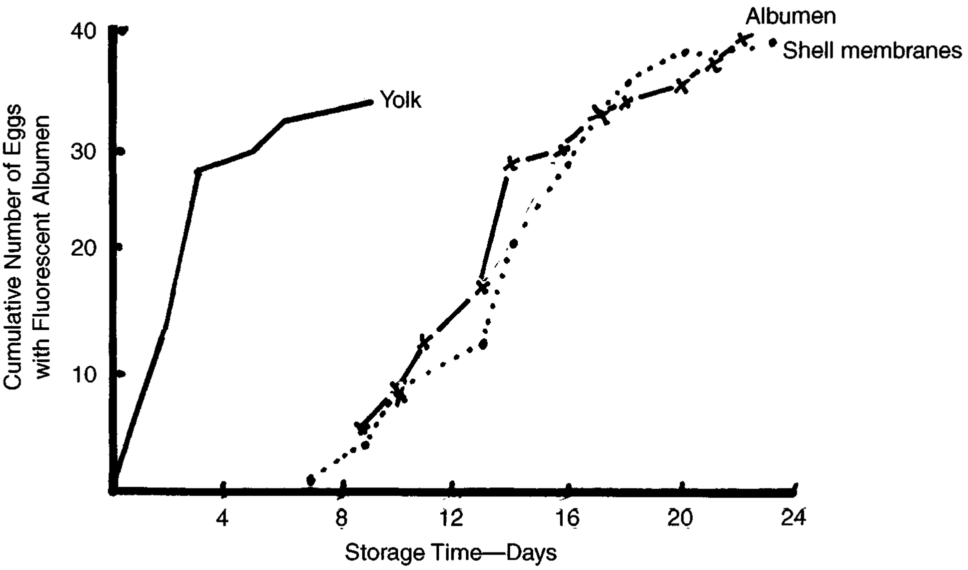

Figure 24-6 Influence of Infection Sites on the Rate of Bacterial Rotting of Hen Eggs. Eggs produced by Single Comb White Leghorn hens were washed on the day following lay in a detergent-sanitizer at 42°C. A washed suspension from an 18 to 24 hour culture of *Pseudomonas aeruginosa* (1.0×10^4 cells) was inoculated into the yolk, shell membrane, or albumen. Inoculated eggs were stored at room temperature and candled with a UV lamp. *Source:* Adapted with permission from W.E. Brown, R.C. Baker, and H.B. Naylor, Egg Spoilage as Affected by the Route of Exposure, *Poultry Science*, Vol. 45, pp. 279–283, © 1966, Poultry Science Association, Inc.

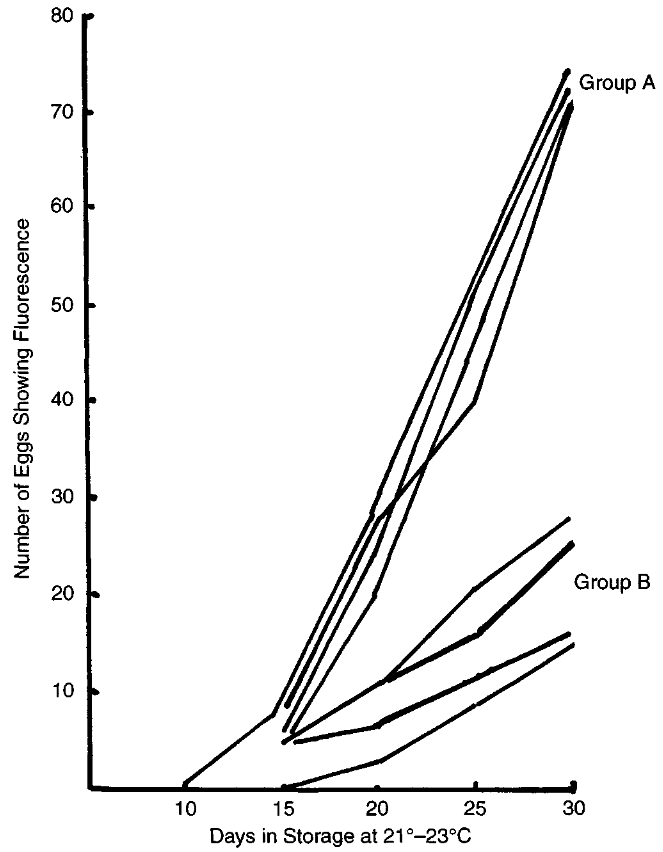

Figure 24-7 Influence of Infection Routes on Bacterial Rotting of Hen Eggs.[142] Eggs laid by White Leghorn hens were washed on the day after lay in a detergent-sanitizer (45°C), rinsed in water (40°C), and allowed to dry at room temperature. The eggs were dipped in paraffin wax (see below), warmed to 35°C, and dipped for 5 minutes in a suspension (1.0 ¥ 10^6 cells ml^{-1}) of *Pseudomonas aeruginosa* in water (15°C). Dipped eggs were stored at 21°–23°C and candled daily with a UV lamp. Very few eggs showed fluorescent whites until about 15 days of storage. Thereafter, the eggs divided into two groups. The eggs in Group A had not been waxed or waxed other than on their broad poles; eggs in Group B had been waxed entirely or on their broad poles. *Source:* Adapted with permission from D.V. Vadehra, Infection Routes of Bacteria into Chicken Eggs, *Journal of Food Science,* Vol. 35, pp. 61–62, © 1970, Institute of Food Technologists.

There is no lag period when there is >2 ppm iron in the inoculum.[15,35] This amount would have no effect on the chelating potential of the ovotransferrin in the albumen. In practice the iron impregnates the glycoprotein mantles on the fibers of the shell membranes, and there is a very slow migration into the underlying albumen.[135] Extensive bacterial growth occurs at the site of iron contamination of the membranes and heavy infection of the underlying albumen ensues.[15]

Seven hypotheses have been put forward to account for the lag period:

1. It is the time required by bacteria to penetrate the shell membranes.[57]
2. Initially, the shell membranes are an inimical environment. Some as yet uncharacterized change in the membranes at about 14 days postlay triggers bacterial growth not only on the membranes but in the underlying albumen.[26]
3. The lag period is terminated in eggs stored at room temperature when the upward movement of the yolk (Figure 24-4) is arrested by an infected shell membrane, leading to rapid bacterial growth.[16,34] This is the most likely interpretation of the data given in Figure 24-7. This particular hypothesis highlights the importance of the albuminous sac as a barrier to yolk movement and the many factors that contribute to its quality including the genetic make up of the hen,[31,132] nutrition,[76] stage in laying cycle,[126] health,[122] and storage conditions[108] of the egg, particularly temperature and relative humidity.[93]
4. The collision of quiescent contaminants of the albumen with the yolks in eggs infected with psychotrophic bacteria and incubated at chill temperatures.[20]
5. The synthesis of siderophores[59] by iron-deprived bacteria in the albumen triggers growth and generalized infection of the egg's contents.[15] If this hypothesis is invoked to explain salmonella growth in albumen, then an explanation is required for the failure to demonstrate siderophore production by coliforms in albumen *in vitro*.[134]
6. Some as yet uncharacterized change in the vitelline membrane accelerates the outward diffusion of nutrients and Fe^{+++} from yolk to white and facilitates bacterial penetration of the yolk.[75]
7. Bacteria in the albumen respond chemotactically to a gradient generated by diffusion of nutrients from the yolk. Some of the bacteria that make contact with the yolk begin to grow, and growth moves progressively outwards until the entire albumen is heavily contaminated with metabolically active, rather than quiescent, organisms some of which invade the yolk causing a generalized infection.[85]

It is noteworthy that the four most plausible hypotheses implicate the yolk in the termination of the lag period. Thus, under commercial conditions, and especially during extended storage at ambient temperatures, the very defense that has evolved to protect the yolk against microorganisms is negated by the yolk.

It is evident from the above discussion that many factors induce multiplication of quiescent bacteria in the albumen. This situation must be recognized by regulatory agencies, especially as no one event can be singled out as the critical factor. Thus, any regulation must recognize the enormous variation in the efficiency of the defense systems of eggs and adopt a storage period that easily accommodates such, and adopt storage conditions, especially chill storage, that ensure maximum retention of the antimicrobial properties of the most vulnerable eggs. In practice the recommendations of the Dick Committee[5] reflect both issues.

24.8 EGG PRODUCTS

In the US in 1992 about 25% (1.5×10^{10}) of all eggs were used to make egg products (total weight approximately 8.0×10^8 kg).[38] It has been predicted that by the year 2000, 50% of all eggs laid in the US will be used for this purpose[8]; the range of products will undoubtedly be large (Tables 24-7 and 24-14). There have been three phases in the evolution of the egg product industry in the US, the largest player in the market. Each had its attendant, and in most cases, unique microbiological problems. A discussion of these phases must take account also of the parallel evolution of the heat exchangers that were required for the dairy industry in order to prepare pathogen-free milk with a long shelf life at chill temperatures.

24.8.1 The Innovative Phase

In 1878, an American firm used a drying process to produce a light brown, meal-like substance from yolk and albumen. Frozen whole eggs (white and yolk not mixed) were prepared commercially in the 1890s.[38] The Maillard reaction between glucose and protein impaired the functional properties of dried egg products.[111] Glucose oxidase or microorganisms, most commonly *Klebsiella pneumoniae,* are used to remove glucose from albumen, whole egg, and yolk prior to drying (Exhibit 24-1). Impairment[39] of the functional properties of yolk stored at ≥ –6°C is prevented by the addition of salt or sugar before freezing (Table 24-7). Such amendments enhance the thermal tolerance of contaminants. This is reflected in the time/temperature combinations that have been adopted to inactivate salmonellas in salted or sugared egg yolk (Table 24-9).

Table 24-7 Major Types of Egg Products and Their Uses

Product	*Examples of Major Use*
Whole egg	
Frozen	Baked goods, institutional cooking, mayonnaise
Dried[a]	Above plus ice cream manufacture; preparation of dry mixes for cakes, etc
Liquid, extended shelf-life products	1 kg cartons or larger amounts in "bag-in-box" packs for use by bakers, caterers, home bakers, etc; shelf life of 28 days with storage at 4°C
Albumen	
Chilled (storage life *ca.* 4 days at 4°C)	
Frozen[a]	Baked goods, icings, chocolate
Dried[a]	
Yolk	
Frozen	
plain[b]	Baked goods, noodles, ice cream
salted	Salad dressings, soups, mayonnaise
sugared	Baked goods, eggnog, ice cream
dried[a]	Dry mixes for cakes, mayonnaise, macaroni
Chilled, liquid with extended life (28 days at 4°C)	1 kg cartons or larger amounts in "bag-in-box" for use by bakers, caterers, home bakers, etc

[a] Glucose must be removed before freezing and drying (methods given in Exhibit 24-1).

[b] There is an increase in viscosity and eventually gelation of plain yolk stored ≥ –6°C. This can be prevented by addition of salt or sugar (commonly at 10% concentration) to yolk before frozen storage.

Source: Data from W.J. Stadelman and O.J. Cotterill, eds., *Food Science and Technology,* © 1995, Food Products Press; and R.C. Baker and C. Bruce, Effect of Processing on the Microbiology of Eggs, in *Microbiology of the Avian Egg,* R.G. Board and R. Fuller, eds., pp. 153–173, © 1994, Chapman and Hall.

Cotterill[38,39] noted that growth in the frozen egg industry in the 1920s and 1930s was not accompanied by much in the way of technical innovation. It was recognized, of course, that the microbiological quality of a product would be determined by the treatment of eggs prior to and immediately following breaking. A regime based on common sense was adopted in order to minimize contamination of products as follows: (1) eggs were candled to eliminate rots; (2) egg shells were washed (sometimes with disinfectants) so that small fragments falling into whites when broken did not cause gross contamination; (3) every egg was broken manually into a small

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Exhibit 24-1 Methods Used to Remove Glucose from Albumen, Whole Egg, and Yolk Prior to Drying

1. **Reasons for and methods of desugaring albumen**
   - Maillard reaction (initially a bonding of glucosidic hydroxyl groups and amine groups of peptides) causes brownish discoloration of dried albumen. Albumen also loses solubility and acquires metallic off-flavor.
   - Glucose can be removed by
     - Spontaneous microbial fermentation—traditional method especially in China, which was used until mid-1940s.
     - Controlled bacterial fermentation (the most commonly used method) of the many organisms studied, a strain of *Klebsiella pneumoniae* has been generally adopted by the industry.
     - Yeast fermentation—3-hour fermentation at 37°C with strains of *Saccharomyces*; eg, *S. cerevisiae.*
     - "Enzyme" fermentation[a]—glucose oxidase used to convert glucose in albumen to gluconate and catalase to break down H_2O_2.
2. **Reasons for and method of desugaring whole egg and egg yolk**
   - Maillard reactions occur (see above); additionally, reaction of glucose with phospholipids, especially cephalin, causes discoloration of dried whole egg or egg yolk powder.
   - Glucose oxidase and catalase system used almost exclusively to remove glucose with incubation at 10°C in order to deter growth of undesirable microorganisms.

[a] For additional details, see Dobbenie et al.[43]

Source: Adapted with permission from M. Sebring, Desugarisation of Egg Products, in *Egg Science and Technology,* 4th ed., W.J. Stadelman and O.J. Cotterill, eds., pp. 323–334, © 1995, Haworth Press.

container and inspected by the egg breakers; 12 or 10 eggs in a larger container were inspected by a supervisor before addition to a bulk tank; and (4) equipment contaminated with a rotten egg was removed from the production line, washed, and sterilized. Such labor-intensive methods, which may well still be practiced in developing countries, were no doubt essential in the days when egg product manufacturers had to buy eggs on an open market supplied by small farms or from chill stores. In the author's experience, this regime produced whole egg of reasonable microbiological quality. Poor plant hygiene led to a pasteurized product that acquired a custard-like character if left overnight at room temperature. This was caused by lecithin breakdown by *Bacillus cereus.* All the faults associated with "bitty cream" formation in pasteurized milk (see Chapter 22) were present in the egg-breaking plant.

As the industry grew, so did the demand for mechanical egg breakers.[40] It is common for such machines to be included in a production line: bulk candling of eggs → washing of egg shells → egg breakers. Inspection of broken-out eggs is commonly done by a person sitting alongside a gully taking the eggs to a bulk tank. It was not uncommon to see eggs with green fluorescent whites go unmolested along the gully. This was due to fatigue and/or boredom of an inspector. The problem was exacerbated also by the need for egg-breaking plants to use eggs that had been stored for long periods. It is evident from the above that flocks of hens dedicated to the needs of an egg processor are required for the preparation of products having low levels of microbial contamination.

24.8.2 Pasteurization

The outbreak of World War II saw a dramatic increase in the demand for dried, whole egg, and it was realized that this product could transmit salmonellas from poultry, which showed no clinical symptoms of infection, to man. Thus, according to a report in 1947, 5 of 43 samples (5-oz packs) of spray-dried egg in the UK intended for home use contained salmonellas. Such packs were often associated with family outbreaks of food poisoning.[3] Many other surveys, reviewed in 1971,[80] showed the incidence of contamination of egg products with salmonellas; for example, 0.3% of 1,649 samples of untreated or chilled liquid egg in England, 2.6% of 786 samples of frozen egg in England, 5% of 39,502 samples of egg products imported into Germany in 1956, and 1.9% of 4,586 samples of liquid egg taken from approximately 30 processing plants in the UK. After the introduction of pasteurization of egg products in the UK, no salmonellas were recovered from 5,980 samples of pasteurized liquid egg or from 11,600 samples of dried egg from the 30 processing plants referred to above.

Pasteurization of whole liquid egg and egg yolk was used first in some parts of the egg product industry in the 1930s.[41] Regulations requiring mandatory pasteurization were introduced in the UK in 1963 and in the US in 1966. Several types of processes are used now to reduce the numbers of microorganisms in liquid egg products (Table 24-8). Many liquid egg products are pasteurized by a high-temperature-short-time (HTST) method, giving a product with a shelf life for whole liquid egg at 4°C of usually up to

7 days, and for salted egg products of up to 4 weeks. An alternative HTST pasteurization method (ultrapasteurization), which was developed in 1985–1987, is used for products that are packed aseptically and are required to have an extended storage life, possibly of 3 to 6 months at 4°C,[9] but in practice often up to 10 weeks. The conditions specified for pasteurization of egg products in the UK, the US, and in the Codex Alimentarius are shown in Table 24-9.

Continuous HTST pasteurization systems heat the liquid egg rapidly to the required temperature and maintain it at that temperature for a specified period of time, by the use of holding tubes of appropriate lengths. The equipment requires control systems to regulate the rate of flow.

The major objective of the heat treatment used for pasteurization was to inactivate the main types of salmonella found in egg products and to minimize the damage to the functional properties of egg. Studies in the 1960s and 1970s of the heat resistance of *Salmonella* spp. in liquid egg indicated that the conditions specified for pasteurization of whole liquid egg would be sufficient to reduce the number of viable salmonellas in the product by 1,000- to 10,000-fold.[47] Rare strains of salmonella occur that would not be destroyed by this treatment. The main strain is *S. senftenberg* 775W, which was isolated from eggs in 1946; among hundreds of isolates from eggs tested in the subsequent 30 years neither this strain nor another strain with comparable heat resistance was found again. In the late 1950s the decision was made that the quality of the pasteurized product should not be sacrificed by the application of a heat treatment that would be required to inactivate a strain that occurred so rarely.[47] The regulations for pasteurization were introduced before *S. enteritidis* became a major problem in eggs, but general experience has been that pasteurization has proved to be effective in preventing the transmission of *S. enteritidis* in egg products.

The heat treatment specified in the UK and in the Codex Alimentarius for liquid whole egg and egg yolk also destroys the activity of α-amylase. This enables a test for α-amylase activity to be used to indicate whether effective pasteurization has been carried out. This enzyme is not inactivated by the minimum heat treatment specified for pasteurization of liquid whole egg in the US (60°C for 3.5 minutes) and no suitable enzyme test is available in the US to determine whether an adequate HTST pasteurization treatment has been applied.[41]

The presence of high concentrations of salt (NaCl) or of sugar (sucrose) in egg products lowers the water activity of the product and protects bacteria against heat treatment, raising their apparent heat resistance. For this reason greater heat treatment is specified for such products. In addition to protecting salmonellas from heat treatment an NaCl concentration as high as 10%, used in some products, would prevent growth of any surviving salmonellas, which would die gradually.[47]

Egg white is much more sensitive to heat treatment than are other egg products; it can be pasteurized at lower temperatures because salmonellas are

Table 24-8 Processes Used To Reduce the Numbers of Microorganisms, Especially Salmonellas, in Liquid Egg Products

Process	*Comments*
Holding method of pasteurization (batch process)	Developed in the 1930s–1940s. Experiments showed that heating whole liquid egg to 57°C, with a 30 min heat-up time, and holding at 57°C for 6 min reduced the number of viable *S. typhimurium* by a factor of at least 10^6.[41] This method is seldom used now in the UK or the US.
High-temperature-short-time (HTST) pasteurization	Times and temperatures shown in Table 24-9. The storage life of liquid whole egg at 4°C is usually 4 to 7 days; that of salted egg products is up to 28 days. This is a major method used for preservation of liquid egg products.
Alternative pasteurization, "ultrapasteurization"	Developed in 1985–1987. This uses higher temperatures than the minimum specified for pasteurization; products are then packed aseptically and the storage life at 4°C is usually up to 10 weeks. This method is used for a significant proportion of egg products in the UK, and its use is increasing.

Table 24-9 Minimum Heat Treatments Specified for Pasteurization of Liquid Egg Products

Product	*Temperature*		*Minimum Holding Time (minutes)*	*Reference*
Whole liquid egg	64.4°C		2.5	36, 131
	60°C	140°F	3.5	139, 140
Egg yolk	64.4°C		2.5	131
	61.1°C	142°F	3.5	139, 140
	60°C	140°F	6.2	139, 140
Egg albumen	57.2–57.8°C		2.5–3.0	131
	56.7°C	134°F	3.5	139, 140
	55.6°C	132°F	6.2	139, 140
Whole egg with 2% more NaCl	63.3°C	146°F	3.5	139, 140
	62.2°C	144°F	6.2	139, 140
Whole egg with 2–12% sucrose	61.1°C	142°F	3.5	139, 140
	60°C	140°F	6.2	139, 140
Egg yolk with 2–12% NaCl	63.3°C	146°F	3.5	139, 140
	62.2°C	144°F	6.2	139, 140
Egg yolk with 2% or more sugar	63.3°C	146°F	3.5	139, 140
	62.2°C	144°F	6.2	139, 140

more heat sensitive at the higher pH of egg albumen than at the lower pH in other egg products. In the past the pH of egg white was usually about 9;[47] the pH can be between 7.6 and 9.3, with a range commonly between 8.4 and 9.2.[8] As a result of the more rapid transport of eggs from the laying site to the processing operation in North America, the pH of commercial liquid egg white is often at or below pH 8.2.[98] This decrease in pH of egg white increases the heat resistance of *Salmonella* spp.; time-temperature treatments for pasteurization of raw egg white at various pH levels have been published.[41]

Alternative processes have been devised for pasteurizing egg white without impairing its functional properties, in particular "whippability," an essential commercial attribute of egg albumen (Exhibit 24-2).

The alkalinity of egg white (commonly pH ≥9.0; Figure 24-4) accentuates heat damage to proteins present and thermal destruction of salmonellas.[33] The majority of the oviductal proteins (Table 24-1) have a maximum stability at pH 7.0. Ovotransferrin (conalbumin) is the exception but its stability is enhanced by the formation of a complex with aluminium. Albumen adjusted to pH 7.0 and supplemented with Al^{4+} requires pasteurization at 60° to 62°C for 3.5 to 4.0 minutes.

Exhibit 24-2 Alternative Methods Proposed for Pasteurization of Egg White without Impairment of Its Functional Properties[a,b]

Lactic Acid plus Aluminum Sulfate Process

Lactic acid is used to reduce the pH of liquid egg white to about 7.0. At this pH the heat stability of most egg white proteins is increased. The major exception is ovotransferrin; this is rendered less heat-labile by the addition of aluminum, forming a complex with ovotransferrin. Egg white treated in this way can be pasteurized in the US at 60° to 62°C for 3.5 to 4 minutes. The process is governed by US government patent; fee-less licenses have been granted to more than 45 firms in the US.

Heat plus Hydrogen Peroxide (Armour Process)

Liquid albumen is heated to not less than 125°F in a plate pasteurizer and maintained in holding tubes for at least 1.5 minutes. Hydrogen peroxide, at least 0.05%, is injected into the liquid albumen, which is maintained at 125°F for a further period of not less than 2 minutes. The product is then cooled and pumped to a drawoff or holding vat, where catalase is added to remove residual hydrogen peroxide. This method is widely used in the US.

Heat plus Vacuum Process

This method requires an HTST pasteurizer equipped with a vacuum chamber. Liquid egg white is maintained in the vacuum chamber for 17–20 minutes before heat treatment at 57°C (134°F) for 3.5 minutes. The vacuum treatment removes air bubbles from the albumen. This method has been approved by the US Department of Agriculture (USDA).

Hot Room Process

Dried, powdered albumen is stored at 53° to 65°C for between 5 and 20 days; this reduces the numbers of salmonellae without impairing functional properties.[c]

[a] Based on Cunningham[41] and USDA.[139]

[b] Lysozyme (for use as an antimicrobial agent in the dairy industry) may be extracted by acidic ion exchange resins before processing. Extraction has a minimal effect on the functional properties of egg white.

[c] Banwart and Ayres.[11]

Storage of dried, powdered albumen at 53° to 65°C for between 5 and 20 days is used to reduce the number of salmonellas without impairing functional properties of the albumen (Exhibit 24-2). Other processes that have been used in the US are also shown in Exhibit 24-2.

Following the general recognition, in the early 1980s, that outbreaks of listeriosis resulted from foodborne transmission of *Listeria monocytogenes,* and that this bacterium can multiply at temperatures of 5°C and lower, attention has been directed to the incidence and survival of *L. monocytogenes* in egg products. *Listeria* spp. are occasional contaminants of egg processing plants and of egg products. Sampling of in-line filters in an egg processing plant on a daily basis for 5 months showed that 125 of 133 samples contained either *L. innocua* (62.2%) or *L. monocytogenes* (37.8%).[88] A mean level of listeria of one per ml of whole egg immediately before pasteurization was reported; no listerias were detected in 500 daily samples of pasteurized egg product. In a nationwide survey in the US of commercially broken, unpasteurized liquid whole egg *L. innocua* was isolated from 15 of 42 samples and *L. monocytogenes* was also isolated from 2 of these 15 samples.[81] The level of contamination with *L. monocytogenes* was estimated as between 1 and 8 cfu ml^{-1}. Following inoculation into raw egg products, a strain of *L. monocytogenes* multiplied in egg yolk at 5°C, showed a slight increase in whole egg, but died out in egg white.[118] At 20°C multiplication of the listeria occurred in egg yolk, whereas in liquid whole egg a 2-log reduction in numbers during the first 24 hours was followed by slow growth. The bacterium died in egg albumen at 20°C. After heating at 121°C for 15 minutes, egg yolk, liquid whole egg, and albumen all supported relatively rapid growth of *L. monocytogenes* at 20°C and at 5°C.

Because of the risk posed by *S. enteritidis* in eggs and the reported incidence of *L. monocytogenes* in egg products, in the 1990s attention has been directed to the effect of heat treatment on the survival of these bacteria in egg products (Tables 24-10 and 24-11).

In a study of 17 strains of *S. enteritidis,* including strains from patients and from egg-associated outbreaks of salmonellosis, after growth at 37°C the D-values at 60°C in liquid whole egg were between 11.8 and 31.3 seconds.[114] The authors stated that on the basis that the true residence time at 60°C for whole liquid egg in a 3.5 minute average holding tube was 2.83 minutes, pasteurization would give a 5.4-D process for their most resistant strain. Sublethal heat shock at 40°C for 2 hours or 48°C for 30 minutes increased the heat resistance 2- or 3-fold. It was calculated that pasteurization at 60°C would give approximately a 4-D kill of *S. enteritidis* with induced thermotolerance, but the authors pointed out that with the use of good manufacturing practices it would be unlikely that thermotolerant salmonellas would be present in commercial liquid egg.[114]

Palumbo et al.[97] used submerged vials to investigate the heat resistance of *Salmonella* spp. and *L. monocytogenes* in liquid egg yolk and egg yolk products; because any lethality during the heating up of the vials apparently was not measured, it would be expected that the D-values reported would be underestimates.

Using a mixture of three strains of *S. enteritidis* and two strains of *S. typhimurium,* grown at 37°C, and an immersed, sealed capillary tube technique, Schuman and Sheldon[109] reported mean D-values in raw liquid egg yolk (pH 6.3) of 0.28 minutes at 60°C and 0.087 minutes at 62.2°C. In egg white pH 8.2 and pH 9.1 the D-values at 56.7°C were 2.96 minutes and 1.54 minutes, respectively.

The results in Table 24-10 indicate that the heat treatments specified in Table 24-9 for pasteurization for whole liquid egg, for egg yolk, and for egg yolk with 10% sucrose should give at least a 4–5 log kill of *S. enteritidis.* The conditions specified for pasteurization of egg yolk with 10% NaCl would have little effect on *S. enteritidis* (or probably on other salmonellas); although these organisms may survive in the product they would be incapable of multiplication and would die slowly. The conditions specified for pasteurization of egg albumen would cause limited inactivation of *S. enteritidis* in egg white at pH 8.2 but would be more effective in the product at pH 9.1. Previous studies showed also that the heat resistance of salmonellas in stored albumen (pH 9.0) was about half of that in fresh albumen (pH 8.0).[37] Recommended pasteurization temperatures for raw egg white at different pH levels have been published[41] or egg white may be aged slightly or the pH adjusted chemically[109] to improve the bactericidal effect of pasteurization.

The results in Table 24-11 indicate that the conditions specified in the US for pasteurization of whole liquid egg and egg yolk may only give a low kill of *L. monocytogenes,* whereas the conditions specified in the UK and by the Codex Alimentarius would be more effective. Conditions specified for pasteurization of products with 10% NaCl would have little effect on *L. monocytogenes.* At high temperatures *L. monocytogenes* may multiply in this concentration of NaCl, and the pasteurized product should be maintained below 5°C, which will prevent this.[48] Pasteurization of egg albumen would have little effect on *L. monocytogenes,* but the organism will tend to die out in this product at high pH.[48]

Table 24-10 Reports Since 1990 of Heat Resistance of *Salmonella* spp., Particularly *S. enteritidis*, in Liquid Egg Products

		Mean D Value (Min) at Following Temperature (°C)											
Organism	*Product*[a]	*55.1*	*56.6–56.7*	*57.2*	*58.3*	*60*	*61.1*	*62.2*	*63.3*	*64.4*	*65.5*	*66.7*	*Reference*
Salmonella enteritidis (17 strains)	LWE			1.2–2.8		0.2–0.52							114
S. enteritidis (4 strains)	EY					0.55–0.75	0.27–0.35	0.21–0.30					97
S. typhimurium (1 strain)	EY					0.67	0.20	0.14					
S. senftenberg (1 strain)	EY					0.73	0.28	0.21					
Mixture of *S. enteritidis* (5 strains) and *S. typhimurium* (1 strain)	EY						0.57		0.20				
	EY + 10% sucrose						0.74		0.72	0.20			
	EY + 10% NaCl								11.50	6.44	3.85	2.07	
Mixture of *S. enteritidis* (4 strains) + *S. typhimurium* (1 strain) + *S. senftenberg* (1 strain)	EW pH 7.8		3.60										98
	EW pH 8.2		2.14										
	EW pH 8.8		1.59										
	EW pH 9.3		1.08										
Mixture of *S. enteritidis* (3 strains) and *S. typhimurium* (2 strains)	EY (pH 6.3)					0.28	0.16	0.087					109
	EW (pH 8.2)	7.99	2.96		1.00								
	EW (pH 9.1)	3.17	1.58		0.52								

[a] LWE = liquid whole liquid egg; EY = liquid egg yolk; EW = liquid egg white.

Table 24-11 Reports of Heat Resistance of *Listeria monocytogenes* in Liquid Egg Products

		Mean D Value (Min) at Following Temperature (°C)										
Organism	*Product*[a]	*55.1*	*56.6–56.7*	*58.3*	*60*	*61.1*	*63.3*	*64.4*	*65.5*	*66.0*	*66.7*	*Reference*
5 separate strains	LWE				1.3–1.7					0.06–0.20		51
2 strains	LWE				1.5, 2.1							14
	LWE + 10% sucrose					1.2, 1.9						
	LWE + 10% NaCl						5.5, 13.7					
	EY + 10% sucrose						0.8, 2.1					
	EY + 10% NaCl						8.2, 18.5					
5 separate strains	EY					0.7–2.30	0.35–1.28	0.19–0.82				97
Mixture of 5 strains	EY					1.41	0.81	0.44				
	EY + 10% sucrose					2.05	1.05	0.97	0.63		0.46	
	EY + 10% NaCl						10.5	6.11	4.30		2.39	
Mixture of 5 strains	EW pH 7.8		10.4									98
	EW pH 8.2		16.5									
	EW pH 8.8		20.3									
	EW pH 9.3		20.9									
Mixture of 5 strains	EY (pH 6.3)				0.28	0.89						109
	EW (pH 8.2)	7.58	4.76	3.47								
	EW (pH 9.1)	7.59	4.35	2.41								

[a] LWE = liquid whole liquid egg; EY = liquid egg yolk; EW = liquid egg white.

Table 24-12 Growth of *Aeromonas hydrophila*, *Listeria monocytogenes*, and *Yersinia enterocolitica* in Pasteurized Liquid Egg Products Stored at the Temperatures Indicated for up to 14 Days

	LWE,[a] pH 6.56, a_w 0.99			EW, pH 9.07, a_w 0.99			LWE + 5% NaCl, pH 7.07, a_w 0.94		
	2°C	6.7°C	12.8°C	2°C	6.7°C	12.8°C	2°C	6.7°C	12.8°C
A. hydrophila	–[b]	+	+	–	–	+	–	–	–
L. monocytogenes	–	–	+	–	–	–	–	–	+
Y. enterocolitica	+	+	+	+	+	+	–	–	–

[a] LWE = liquid whole egg; EW = egg white.

[b] + = growth; – = no growth.

Source: Data from J.P. Erickson and P. Jenkins, Behaviour of Psychotrophic Pathogens *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Aeromonas hydrophilia* in Commercially Pasteurized Eggs Held at 2, 6.7 and 12.8°C, *Journal of Food Protection*, Vol. 55, pp 8–12, © 1992, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES)

It should be emphasized that there appear to have been no reported outbreaks or cases of listeriosis associated with egg products; this may be because the incidence and numbers of *L. monocytogenes* in these products are low and in those products where pasteurization has least effect on listerias any surviving organisms are unable to multiply. The other psychrotrophic pathogenic bacteria, *Yersinia enterocolitica* and *Aeromonas hydrophila,* are less heat resistant than *L. monocytogenes* and are, therefore, less likely to survive pasteurization of egg products. These three psychrotrophic bacteria can multiply in some pasteurized egg products stored at low temperature, depending on the pH and the salt content of the product[48] (Table 24-12).

The unsalted egg products in Table 24-12 all showed spoilage, due to pseudomonads, or to *Serratia* spp. and coliforms, in 10–14 days; this might prevent the use of products in which growth of pathogens had occurred. The liquid whole egg containing 5% NaCl stored at 2°C and 6.7°C was not spoiled in this time, but after storage at 12.8°C for 14 days spoilage due to fecal strepococci and micrococci was reported. Thus, post-pasteurization contamination of egg products could result in multiplication of psychrotrophic, pathogenic bacteria, but with properly controlled refrigerated storage at ≤5°C for 5 to 10 days and hygienic conditions the risk was judged to be minimal.[48]

The adoption of pasteurization of whole liquid egg killed many spoilage bacteria as well as pathogens, giving a reduction in viable count of the order of 5 × 10^3 g^{-1}.[100] This caused an important change in the industry in that chilled liquid egg could be delivered directly to the bulk users, reducing the need for freezing or drying egg products. Pasteurized whole liquid egg was rarely, if ever, available in small packs for use in the home or institution, but this form of marketing became feasible with the introduction of alternative methods of pasteurization at higher temperature.

An alternative pasteurization procedure, termed by the originators of the process "ultrapasteurization," was developed in the mid-1980s. The process used high-temperature-short-time heat treatment at temperatures between 63.7°C and 72.2°C followed by aseptic packaging, and aimed to provide a product with an extended shelf life, under refrigeration, of 3 to 6 months and with an acceptable functional quality.[9] The most severe heat treatments used by Ball et al.[9] were equivalent to heating at 71.5°C for 36.5 seconds or at 71.8°C for 62.5 seconds. These treatments were reported to give shelf lives of at least 20 to 24 weeks at 4°C. Typical temperatures and holding times used for ultrapasteurization are shown in Table 24-13. In the UK a significant proportion of liquid egg products is produced by ultrapasteurization and the use of this process is increasing. In order to produce a satisfactory final ultrapasteurized

Table 24-13 Typical Temperatures and Times Used for Alternative Pasteurization (ultrapasteurization) of Liquid Egg Products

Product	Temperature (°C)	Holding Time (Minutes)
Liquid whole egg	69	1.5
Liquid yolk	65.5	5.0
Albumen	57	5.0

Table 24-14 Value-Added Egg Products[a]

Product	*Comments*
Hard-cooked eggs	Hard-boiled eggs have shells removed manually or mechanically and are packed (sometimes following immersion in boiling water or exposure to steam) in an acid solution containing a preservative *viz.* 0.2%–0.85% citric acid with 0.1% benzoate. Packed eggs are stored at approximately 0°C. Alternatively the eggs can be repacked after 4 days in a fresh solution of citric acid and benzoate and heat treated (boiling water for 20 minutes or steam at 121°C for 15 minutes). Stadelman et al.[129] found yeasts and mold growth on hard-cooked eggs after storage at 4° or 22°C for 1 week. Oblinger and Angalet[92] reported rapid bacterial growth and slime production in hard-cooked eggs stored at 25°C.
Diced egg[b]	Eggs broken into large trays, cooked in steam, diced while hot, and packed hot (82°C). Very limited microbial contamination, hence long shelf life at chill temperatures.
Hard-cooked egg[b] roll	Yolk surrounded by white in a polypropylene tube is cooked at 93°C for 20 min. Apparently, there are no microbiological problems during storage.
Scrambled eggs[b]	Eggs are scrambled, cooked, and packed hot (82°C) in appropriate containers for institutional use. The shelf life at the recommended storage temperature of 2°C is upwards of 45 days. It has been claimed that with use of pasteurized whole egg and Good Manufacturing Practice (GMP), there are no problems due to bacterial contaminants.
Frozen omelettes	Omelettes (either plain or filled) are sealed in plastic packs and frozen by cryogenic techniques in order to prevent ice-crystal damage to product.
Low-fat, low-cholesterol egg yolk[c]	Fat and cholesterol extracted from dried egg yolk using supercritical CO_2. As it is made from USDA certified pathogen-free ingredients and is manufactured with GMP under USDA supervision, problems with bacterial pathogens will be a very low risk.

[a]Development of value-added products discussed by Baker and Bruce[8] based on Stadelman.[127]
[b]Baker and Bruce.[8]
[c]Bringe and Cheng.[25]

product with ≤10 bacteria ml^{-1}, it is necessary to use good quality eggs that are very fresh and to pay critical attention to the cleanliness and hygiene of processing equipment. In the studies by Foegeding and Stanley[52] *Bacillus circulans* and *Enterococcus faecalis* were the most heat-resistant bacteria isolated from ultrapasteurized egg.

24.8.3 Value-Added Egg Products

A selection of value-added egg products is listed in Table 24-14. In practice, these commodities can be considered to be basically cooked or partially cooked high-protein items. As there is as yet little information about the storage history of such items it would appear sensible to adopt Good Manufacturing Practice developed for the preparation, distribution, and storage of cooked meat products.

24.9 THE FUTURE

The above discussion of ultrapasteurized and value-added egg products highlights the possible role of eggshells in transporting salmonellas from the laying hens to the processing plant. From a review of the literature, Humphrey[71] reported the following incidences of *Salmonella* contamination of the shells of eggs associated with human cases of food poisoning: 0% of 760, 1.1% of 372, 5% of 68, and 5.2% of 194 eggs. An incidence of shell contamination of 0.5% was reported for 998 eggs not associated with food poisoning. At first sight, these incidences might be considered to be insignificant. They are not, however, when considered in the context of the vast number of eggs used by the manufacturers of egg products. Indeed, it is evident that egg reception areas ought to be physically separated from egg processing

Exhibit 24-3 Practices with Potential To Bolster and/or Compromise the Safety of Ultrapasteurized or Value-Added Egg Products

Practices having the potential to bolster safety

1. Selection of layers that produce a well-defined albuminous sac, a low incidence of shell faults, and a high incidence of deposition of quality cuticle over the entire surface of an egg shell.
2. Use of eggs from dedicated flocks housed under conditions that minimize exposure to salmonellas.
3. Choice of an appropriate egg-washing regimen and the selection of detergent/sanitizers that do not damage cuticle integrity but effectively kill salmonellas on shell surfaces and impede cross-contamination.[33,42,49,78]
4. Pulsed light (8 flashes at 0.5J cm^{-2} per flash (for total treatment of 4J cm^{-2}) gave an 8-log kill of *Salmonella enteriditis* on surface of egg shell.[46]
5. Pasteurization of intact shell eggs.[70,130] A combination of heating whole eggs in a water bath (57°C for 25 minutes) and hot air oven (55°C for 60 minutes) gave a 7 log reduction in the numbers of *Salmonella enteriditis* in deliberately inoculated eggs.

Practices having the potential to compromise safety

1. Washing warm eggs in cold water containing ≥ 2 ppm Fe^{+++}.
2. Using the contents of eggs with cracked shells.[141]
3. Using eggs that have been stored for unknown periods at unknown temperatures.
4. Inadequate separation of egg reception (the dirty area) from egg processing (the clean area).

in plants producing ultrapasteurized or value-added egg products. Such separation ought to go some way toward guarding the finished product from cross-contamination. In addition, some or all of the factors listed in Exhibit 24-3 might well be considered.

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25 Fresh and Processed Vegetables

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25.1 INTRODUCTION

Vegetables and vegetable-based foods are normal parts of the human diet and are consumed daily in large quantities in most civilizations. Vegetable crops are grown in environments widely open to multiple sources of microbial contamination, the main sources being air, soil, and water. The influence of microorganisms on vegetables is part of everyday life. Spoilage of vegetables is visible daily in domestic refrigerators. Large-scale microbial spoilage had tragic and historic consequences. Thousands of Irish people died from starvation or left Ireland for America in the mid-nineteenth century, after the massive and disastrous attack on potatoes by the fungus *Phytophthora infestans*. Warnings about the consumption of raw vegetables in developing countries and reports of outbreaks show that vegetables may transmit human pathogenic microorganisms. Nevertheless, the majority of vegetables consumed are sound and free from hazards.

After harvest, vegetables can be considered as food products, but fresh vegetables continue to be living plant tissue. This tissue is compartmentalized by structures that limit the diffusion of solutes and this influences the survival and growth of microorganisms. In considering the interactions between microorganisms and vegetables, this compartmentalization must be taken into account. Vegetables may contain chemical compounds that inhibit microorganisms and may also produce defense reactions, similar to those formed by growing plants, against microbial invasion.

Vegetables present contrasting features, depending on the botanical organs. The post-harvest life of leaves, flowers, or fruits normally leads to senescence in contrast to that of storage organs like bulbs, tubers, and roots. Vegetables are also processed to make them more palatable or more stable than the raw ones. This processing has a lethal effect on vegetable tissue and modifies the microflora of vegetables and its behavior. Some specific aspects of the microbiology of processed cooked, dehydrated, and frozen vegetables will be considered.

This chapter will give an account of the diverse microflora of vegetables, the biological relations with the plant organ, and illustrate the consequences on the quality and safety.

25.2 THE MICROBIOLOGY OF RAW VEGETABLES

25.2.1 Numbers and Types of Microorganisms on Raw Vegetables

Numbers of Microorganisms

The number of aerobic mesophilic bacteria reported on vegetables varies between 10^2 and 10^8 cfu g^{-1} (Table 25-1). It varies among the various kinds of vegetables; the highest counts were found on salad sprouts and the lowest on vegetable fruits or on the inner leaves of cabbage heads. Counts are also extremely variable among samples from the same veg-

Table 25-1 Numbers of Aerobic Mesophilic Bacteria Found in Fresh Vegetables

Vegetable	*Place of Sampling*	*Aerobic Mesophilic Bacteria (log cfu g^{-1})*[a]	*Incubation Temperature of Media (°C)*	*Reference*
Leafy vegetables				
White cabbage	Field	2.7 to 5.0 for outer leaves	10 + 20	202
	Retail	6.0	28	281
Cabbage	Field and retail[b]	4.0 to 8.0 for 82% of samples	37	197
Collard leaves	Field	6.5 to 6.8	20	455
	Field and retail[b]	5.5 to 6.5	28	487
Brussels sprouts	Field and retail[b]	4.0 to 6.0	37	197
Iceberg lettuce	Retail	6.1	25	280
	Retail	4.0 to 5.0	30	73
Lettuce	Field and retail[b]	4.0 to 8.0 for 92% of samples	37	197
	Retail	5.0 to 8.6	30	4
	Retail	5.4 to 6.0	30	179
	Field	3.0 to 6.0	22	382
Green endive	Field	5.0 to 6.0	25	369
	Factory processing vegetables	6.0 to 8.0	30	219
	Field and retail[b]	6.0 to 8.0	37	197
Witloof chicory	Retail	4.0	NS	31
	At production (hydroponic)	5.0[a]	28	507
Spinach	Factory processing vegetables	6.0	30	198
	Field and retail[b]	4.0 to 8.0 for 95% of samples	37	197
Celery	Field and retail[b]	4.0 to 8.0 for 85% of samples	37	197
	Field	4.9 to 6.4	22	426

continues

Table 25-1 Continued

Vegetable	*Place of Sampling*	*Aerobic Mesophilic Bacteria (log cfu g^{-1})*[a]	*Incubation Temperature of Media (°C)*	*Reference*
Flower vegetables				
Cauliflower	Field and retail[b]	4.0 to 6.0 for 56% of samples	37	197
Artichoke	Field and retail[b]	4.0 to 8.0 for 88% of samples	37	197
Broccoli	Wholesale distributor	5.2 to 5.6	25	364
	Field	6.5	30	87
Roots, tubers, and bulbs				
Carrots	Factory processing vegetables	6.0	32	502
		6.8	30	198
	Retail	6.0	30	72
Potatoes	NS	4.8 to 3.5	NS	473
Fennel	Retail	4.1 to 4.7	30	179
Fruit vegetables				
Tomatoes	Packing house	4.0 to 5.7	20	456
	Retail	4.0	30	74
	Retail	5.0 to 6.0	30	437
	Field	4.8	30	86
	Retail	3.0	35	425
Cucumber	Retail	5.0	35	425
Aubergine	Retail	3.0	35	425
Courgette	Retail	7.0	35	425
Green pepper	Retail	4.0	35	425
Bell pepper	Field	5.5	30	88
Pods, peas, and beans				
Green peas (shelled)	Factory processing vegetables	≥7	20	457
	Retail	2.3 to 2.5	28	487
	Field	6.0	28	487
Green beans	NS	5.8 to 6.5	NS	473
Field sprouts				
Asparagus	Field and retail[b]	4.0 to 6.0	37	197
Hydroponic sprouts				
Various salad sprouts	At production	7.0 to 8.0	30	477
Mung bean sprouts	At production	7.5	30	510
		8.0	30	358
	Retail	7.0	8	407

[a] Or log cfu cm^{-2}.

[b] No differences between samples from field and samples from retail market.

NS = Not stated.

etables. Variations within a survey, among samples of the same vegetables, may reach 4 log units.[197,487] It is therefore difficult to draw general features.

This variability may reflect the great diversity of conditions prevailing during cultivation and post-harvest life of vegetables. The high counts found on shelled green peas[457] may result from post-harvest contamination. However, hydroponically grown vegetables are not less contaminated than field grown products (Table 25-1) and numbers of bacteria on vegetables sampled at retail were not different from those sampled in the field.[197] Counts are usually obtained on general media incubated between 20° and 30°C, except for some authors[197] who incubated media at 37°C. This was probably above the optimum growth temperature of many bacteria from vegetables and could have led to a slight underestimation of the total number of bacteria. For instance, among incubation temperatures ranging from 4° to 35°C the highest number of bacteria from cabbage leaves were counted on plates incubated at 20°C.[281] On celery[426] and lettuce[382] bacterial counts from plates incubated at 22°C exceeded those from plates incubated at 37°C by 1 or 2 log cyles. The optimum growth temperature of strains of pseudomonads isolated from vegetables was between 25° and 30°C.[92]

Types of Microorganisms

The majority of Gram-negative rods identified from raw vegetables were fluorescent pseudomonads, *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., *Flavobacterium* spp., *Xanthomonas* spp., *Chromobacterium* spp., and *Alcaligenes* spp.[86–88,281,358,364,473,507] Gram-negative rods were predominant among isolates from a range of vegetables[473] such as broccoli,[364] cabbage,[281] endive,[507] mungbean sprouts,[358] and carrots.[502] An important proportion of isolates from broccoli were coryneform bacteria[87] and catalase-negative cocci predominated in raw peas and raw sweet corn.[473] In India, the mesophilic microflora of potatoes mainly comprised Gram-positive bacteria, *Bacillus* spp., *Micrococcus* spp., and *Sarcina* spp.[210]

Selective and diagnostic media have been used to differentiate the main groups of bacteria found on vegetables (Table 25-2). Selective media for enterobacteria may prevent growth of some strains and underestimate their numbers as found on tomatoes[86]; counts on selective medium were 2 log units lower than on the nonselective medium for mesophilic bacteria, whereas enterobacteria represented at least 10% of the mesophilic bacteria counted on the nonselective medium. Pectolytic microorganisms are frequently enumerated to estimate the number of microorganisms that could cause spoilage, because the enzymic breakdown of pectic polymers in the plant cell wall is the main cause of maceration and soft rot development.[315] Pectin degradation is revealed on pectin-based media, either by chemical reagents[443] or by the formation of pits in the pectate gel.[141,393] Pectolytic bacteria have been identified as fluorescent pseudomonads, *Cytophaga* spp., *Flavobacterium* spp., *Xanthomonas* spp., and *Erwinia* spp. Among lactic acid bacteria, *Leuconostoc mesenteroides* was the most frequent and the most abundant species found on vegetables (Table 25-2). A majority of isolates (70%) produced slime on sucrose medium,[374] a trait that may be important in spoilage of some processed vegetables.

The number of yeasts and molds found on various raw vegetables was between 1 and 5 log cfu g^{-1},[86,87,202,280,281,455,456,457] although cfu is probably not appropriate to quantify molds. Yeasts identified belonged to the genus *Cryptococcus, Candida, Rhodotorula,* and *Sporobolomyces*.[148,202] Molds belonged to the genus *Cladosporium, Aureobasidium, Aspergillus, Penicillium, Phoma, Botrytis, Fusarium, Epicoccum,* and *Geotrichum*.[78,202]

Microorganisms of Public Health Significance

Information about bacterial indicators of fecal contamination on raw vegetables is abundant, but most data concern fecal coliforms cultivated at 44°C. Their value as fecal indicators is poor[89,317,473] because many bacterial species, normally present on the surface of vegetables, give positive results on media for fecal coliforms. For instance, on carrots, only 2% of isolates from plates for fecal coliforms (incubated at 44°C) were identified as *E. coli*, while the majority of isolates were identifed as *Enterobacter aerogenes, E. amnigenus, E. sakazakii,* and *Klebsiella pneumoniae*.[502] Similarly, on various vegetables[275,276] and on bean sprouts,[358,477] coliforms were predominantly *Klebsiella* spp. *Escherichia coli* is considered as a better indicator of a possible fecal pollution.[216] Most surveys found low numbers of *E. coli*[216,425,426,494] (Table 25-3). Higher levels of contamination with *E. coli* (>10 per g) were found on vegetables imported from tropical countries,[494] on bean sprouts and cress in England[425] and in some samples in Spain.[197] Presence of *E. coli* on raw vegetables was associated with a higher incidence of *Salmonella* in some studies[207,494] but in a survey of over 345 samples in Spain[197] a third of the vegetables positive for *Salmonella* did not contain detectable *E. coli*. In the United States (US), Stewart et al.[487] also failed to find a relation between *Salmonella* and *E. coli* on raw vegetables.

The major agents of foodborne diseases have been detected on vegetables (Table 25-3). Most vegetables were sampled at retail and contamination with pathogenic microorganisms may have occurred after harvest. *Aeromonas hydrophila*[104,248] and the spore formers *Bacillus cereus* and *Clostridium perfringens*[425] were detected in a third or more of raw vegetable samples. The incidence of other pathogenic bacteria on vegetables was lower than 10% in most surveys carried out in developed countries and the bacteria were detected only after enrichment proce-

Table 25-2 Groups of Bacteria, Enumerated on Selective or Diagnostic Media, from Raw, Unprocessed Vegetables

Vegetables	*Microorganisms*	*Media, Temperature (°C)*	*Number (log cfu g^{-1})*	*% of Aerobic Mesophilic Flora*	*Reference*
Bell pepper	Enterobacteria	VRBG, 32	4.0	3.2	88
Tomato		VRBG, 35	<1 to 3.7	≤1	456
		VRBG, 32	2.8	1	86
Broccoli		VRBG, 32	5.8	25	87
Collard		VRBG, 35	5.3 to 5.6	3 to 10	455
Peas		VRBG, 35	4.6	<1	457
Bean sprouts		VRBG, 30	8	63	358
Salad sprouts		VRB, 35	7	15	477
Green endive	Fluorescent pseudomonads	KB, 25	2.9 to 4.8[a]	0.5 to 12	369
		KB, 37	6.4	3.2	216
		KB, 25	4.7 to 6.8[a]	14 to 78	254
White cabbage		KB, 10+20	0.8 to 4.6[a]	0.2 to 40	202
Lettuce		KB, 22	2.0 to 4.0	NS	382
Celery		KB, 25	1.9 to 3.0	<0.01 to 0.6	426
White cabbage[b]	Pectolytic bacteria	Paton, 10+20	0.4 to 4.1[a]	0.05 to 32	202
Lettuce		Paton, 25	3	NS	382
Green endive		Paton, 25	3.2 to 4.[a]	1 to 35	254
		Paton, 25	2.3 to 3.6[a]	0.04 to 0.5	369
Celery		Paton, 25	3.0 to 3.7	0.06 to 2.5	426
Potatoes[c]		Stewart, NS	3.5 to 3.9	0.01	210
Various vegetables	*Leuconostoc mesenteroides*	MRS + azide, 32	4.4[d]	NS	374
	Lactobacilli	MRS and Rogosa, 32	1.6[d]	NS	373
Tomatoes	Lactic acid bacteria	Rogosa, 30	2.5	0.5	86
White cabbage	Psychrotrophic bacteria	PCA, 8	6.2	68	281
Carrots		PCA, 7	6.5	100	72
Iceberg lettuce		PCA, 7	2.5 to 4	32 to 100	73
Tomatoes		PCA, 5	3.2	2.5	86
Broccoli		PCA, 5	3.8	0.2	87

[a] log cfu cm^{-2}.

[b] Outer leaves only.

[c] Periderm only.

[d] Mean counts of samples in which the bacterium was detected (ie, 53% of samples for *L. mesenteroides*, 29% for lactobacilli).

NS = Not stated.

VRBG = crystal violet—neutral red—bile salt—glucose—agar, KB = King B medium, MRS = De Man Rogosa and Sharpe medium, PCA = plate count agar.

dures (Table 25-3). However, some publications reported a high incidence of *Listeria monocytogenes* on potatoes and radishes in the US,[231] on bean sprouts in France,[399] and a high incidence of *Salmonella* on lettuce and fennel.[179] In surveys done on various foods, vegetables are usually less contaminated with foodborne pathogens than foods of animal origin[377]: *L. monocytogenes* was not detected in a range of vegetables in Canada[183] whereas it was detected in 26% to 80% of raw meat products. Vegetables may also harbor other microorganisms that are potential pathogens for humans, such as *Pseudomonas aeruginosa*.[218,285,534]

25.2.2 The Effect of Microorganisms on Quality and Safety of Vegetables

Many microbial species cause spoilage of harvested raw vegetables (Table 25-4). In the US, losses due to post-harvest diseases were estimated as 7% to 11% of the crop for tomatoes, 4% for bell peppers, 1% to 3% for potatoes, and 2% to 3% for iceberg let-

Table 25-3 Incidence of Microorganisms of Public Health Significance on Raw, Unprocessed Vegetables

Vegetables	*Place of Sampling*	*Number of Samples*	*Microorganism*	*Incidence (%)*	*Number (cfu g^{-1})*	*Reference*
Various vegetables	Retail (US)	21	*Aeromonas* spp. (cytotoxic & hemolytic)	99	$<10^2$ to 10^4	104
Lettuce, endive, watercress	Retail (Brazil)	90	*Aeromonas hydrophila, A. caviae*	47.8	10^2 to 10^6	248
Bean sprouts	At production (US)	16	*Bacillus cereus*	12.5	10^4	477
Seeds for salad sprouts	Retail (US)	98	*B. cereus*	69[a]	3 to >500	227
Various vegetables	Retail (UK)	100	*B. cereus*	35	>0.04	425
Mushrooms (packaged)	Retail (US)	200	*Campylobacter jejuni*	1.5	>0.04	164
Various vegetables	Outdoor markets (Canada)	533	*Campylobacter* thermotolerant	1.7	>0.04	390
	Supermarket (Canada)	1031	*Campylobacter* thermotolerant	0	<0.04	
Various vegetables	Retail (UK)	100	*Campylobacter* spp.	0	<0.04	425
Cabbage	Retail (US)	88	*Clostridium botulinum* (type A)	13.6	NS	469
Mushroom	At production (Netherlands)	50	*C. botulinum*	0	<0.01	384
Onion	Restaurant (US)	75	*C. botulinum* (type A)	6.7	NS	467
Garlic	NS	115	*C. botulinum*	4.3	NS	468
Various vegetables	Retail (UK)	100	*Clostridium perfringens*	34	>0.04	425
Bean sprouts	At production (UK)	104	*Escherichia coli*	38	NS	386
Iceberg lettuce	Retail (Norway)	42	*E. coli*	0.4	10 to 10^4	271
Celery	At production (UK)	90	*E. coli*	50	0.001 to 0.004	426
Various vegetables	Retail (UK)	100	*E. coli*	14	0.3 to 460	425
	At production and retail[b] (Spain)	345	*E. coli*	86	>10 for 83% of positive samples	197
	Retail (Netherlands)	260	*E. coli*	26	0.03 to 1	494
				22	10 to $>10^6$[c]	
	NS (Mexico)	89	*E. coli* 0157:H7	19	NS	540
Unspecified raw vegetables	Retail (UK)	64	*Listeria monocytogenes*	6.2	0.04 to 50	328
Leafy vegetables	Retail (Malaysia)	22	*L. monocytogenes*	22	>0.05	29
Bean sprouts	Idem	7	*L. monocytogenes*	85	Idem	29
Iceberg lettuce	Retail (US)	22	*L. monocytogenes*	9	NS	486
Lettuce, endive	Raw material for processing (France)	89	*L. monocytogenes*	0	<0.3	216
Various vegetables	Retail (Germany)	103	*L. monocytogenes*	2	>0.1	91
	Retail (Canada)	110	*L. monocytogenes*	0	<0.1	183

continues

Table 25-3 Continued

Vegetables	*Place of Sampling*	*Number of Samples*	*Microorganism*	*Incidence (%)*	*Number (cfu g^{-1})*	*Reference*
Potatoes and radishes	Retail (US)	264	*L. monocytogenes*	25 to 30	>0.04	231
Other vegetables	Idem	736	*L. monocytogenes*	0 to 2	Idem	231
Unspecified	NS (UK)	279	*L. monocytogenes*	9	NS	353
Various vegetables	Retail (Spain)	103	*L. monocytogenes*	7.8	>0.04	147
	NS	20	*L. monocytogenes*	0	<0.04	500
	Retail (UK)	108	*L. monocytogenes*	1.8	>0.04	513
Bean sprouts	At production (France)	31	*L. monocytogenes* 4b	3.1	0.04 to 100	358
	Retail (France)	102	*L. monocytogenes*	19	0.04 to 100	399
Various vegetables	At production (US)	1120	*Pseudomonas aeruginosa*	0.1	NS	218
Bean sprouts	At production (US)	13	*Salmonella*	0	<0.02	477
	At production (France)	31	*Salmonella*	0	<0.04	358
Iceberg lettuce	Retail (Norway)	57	*Salmonella*	5.3	NS	271
Lettuce and fennel	Retail (Italy)	209	*Salmonella* (various serotypes)	69.9	0.01 to 0.4	179
Various vegetables	Retail (US)	50	*Salmonella* (various serotypes)	8	NS	430
	Retail (UK)	100	*Salmonella*	1	>0.04	425
	At production and retail[b] (Spain)	345	*Salmonella* (various serotypes)	7.5	>0.2	197
	Retail (Egypt)	71	*Salmonella*	2.8	>0.04	437
	Retail (Netherlands)	103	*Salmonella*	22.3	<1[e]	494
	At production and retail[b] (Spain)	849	*Salmonella* (various serotypes)	1.7 to 8.3	>0.2	196
Bean sprouts	Retail (Thailand)	344	*Salmonella* (various serotypes)	8.7	>0.04	258
Iceberg lettuce	Retail (Norway)	57	*Shigella sonnei*	0	NS	271
Various vegetables	Retail (UK)	100	*Shigella* spp.	0	<0.04	425
	Retail (Egypt)	71	*Shigella* spp.	2.8	>0.04	437
Bean sprouts	At production (US)	10	*Staphylococcus* spp. (coagulase positive)	10	10^4	477
Various vegetables	Retail (UK)	100	*Staphylococcus aureus*	0	<0.04	425
Various salad sprouts (packaged)	Retail (US)	54	*Staphylococcus aureus*	24	>100	407
Various vegetables	Retail (UK)	100	*Vibrio* spp.	0	<0.04	425

continues

Table 25-3 Continued

Vegetables	*Place of Sampling*	*Number of Samples*	*Microorganism*	*Incidence (%)*	*Number (cfu g^{-1})*	*Reference*
Unspecified vegetables	Retail (Italy)	16	*Yersinia enterocolitica*[d]	12.5	>0.04	69
Various vegetables	Retail (UK)	100	*Y. enterocolitica*	0	<0.04	425
	Retail (UK)	65	*Y. enterocolitica*[d]	3.2	>0.1	149
Leafy vegetables	Retail (Brazil)	30	*Y. enterocolitica*[d]	3.3	<0.04	161
Unspecified vegetables	NS	86	*Y. enterocolitica*[d]	43	>2.5	144
Leafy vegetables	Retail (Brazil)	200	*Ascaris* eggs	17.5	0.1 to 0.3	146
Various vegetables	At production and retail (Korea)	1305	*Ascaris* eggs	22 to 96	0.002 to 0.04	129
	Retail (US)	568	*Ascaris* eggs and larvae	3.3	NS	430
		276	Active amoebic forms	38.4	NS	430
		292	*Giardia* spp.	0	NS	430
	Retail (Costa Rica)	640	*Cryptosporidium* oocysts	2.8	NS	366
	Gardens (Brazil)	NS	*Giardia* spp.	13	NS	341

[a] Contaminated seeds gave sprouts with numbers of *B. cereus* ranging from $<10^3$ to $>10^6$ cfu g^{-1}.

[b] No differences between vegetables sampled at production and at retail.

[c] Vegetables imported from tropical countries.

[d] Serotypes not pathogenic to man.

[e] One sample with 240 *Salmonella* per g.

NS, not specified.

tuce.[230] Occasionally, some microorganisms may be devastating, for example, 60% of a tomato shipment was destroyed by *Mucor mucedo* in the US.[365]

Microorganisms responsible for post-harvest diseases are not necessarily dominant on the surface of sound vegetables: on cabbage leaves *Botrytis cinerea* represented less than 0.1% of the total microflora[202] and on witloof chicory only 3% of the epiphytic isolates caused spoilage upon inoculation.[507] Some spoilage microorganisms may be specific to a few vegetable species whereas others, such as *Botrytis cinerea*, *Sclerotinia* species, *Sclerotium rolfsii*, *Rhizopus stolonifer*, or soft rot bacteria, may attack a wide range of products, the examples given in Table 25-4 are only indicative. The difference between spoilage agents able and those unable to spread during storage (Table 25-4) is of great practical significance. In the first case, losses would increase during the storage period, whereas in the second case, potential losses would be a direct consequence of pre-harvest conditions. The relative importance of spoilage organisms for a given vegetable may differ in different countries and climates. *Mycocentrospora acerina* and *Phythophthora megasperma* were the main cause of spoilage of stored carrots in Normandy, France,[300] whereas in England only *Botrytis cinerea* and *Rhizoctonia carotae* were significant agents of spoilage.[204] In Denmark *R. carotae* was the major cause of post-harvest decay.[257] On freshly harvested cabbage, *Alternaria tenuis* was the main cause of spoilage, *B. cinerea* prevailed on cabbage stored under air, and *Fusarium roseum* was an important cause of spoilage for cabbage stored under modified atmosphere.[3] Some microbial species do not cause spoilage of raw vegetables, but may have an important impact on the quality of processed vegetables. This will be discussed later.

Significant proportions of strains of *Alternaria alternata* and *Fusarium* spp. produce mycotoxins in decaying tomatoes[488] or potatoes.[278,279] Spoiled vegetables would not be consumed directly but traces of spoiled vegetables may be incorporated into processed products. However, a recent report concluded that vegetable products do not represent a significant hazard with regard to mycotoxins in European countries.[464] The situation could be different in de-

Table 25-4 Microorganisms That Cause Post-Harvest Spoilage of Vegetables

These microorganisms cause a large amount of post-harvest spoilage of vegetables, many of them also cause disease or spoilage of vegetables in the field, before harvest. More detailed information is available in references.[151,466]

Microorganisms	*Vegetable*	*Symptoms*	*Mode and Stage of Contamination*	*Reference*
Fungi				
Alternaria alternata and *Alternaria* spp.	Cucurbits Tomatoes, pepper, aubergine Green beans Brassica Potato	Dark lesions	Infection before harvest through injuries, cracks, flower or stem scars, direct penetration of leaves and lenticels	3, 78, 79, 466
Alternaria dauci and A. radicina	Carrot	Black lesions	Infection before harvest from leaves or from injuries of the root	466
Alternaria porri	Onion, leek	Dark rot of bulb	Infection at harvest at the neck site of bulbs or in wounds	466
Ascochyta spp.	Green beans and peas	Dark sunken lesions	Infection in the field through damaged tissue and stem end	466
Aspergillus niger	Onion, garlic	Black mold on the surface of bulbs	Infection via the neck of bulb at harvest through senescent foliage	41, 466
	Yam	Brown firm rot	Infection via wounds at or after harvest	
Botriodiplodia theobromae	Cucurbits Cassava Taro Sweet potato Yam	Wrinkle lesions, soft or dry rot	Wound infection—warm climate	466
Botrytis allii and B. squamosa	Onion	Soft rot	Infection of bulb via the neck through infected leaves	466
Botrytis cinerea	Cucurbits Tomato, pepper, aubergine Green beans and peas Brassica Artichoke Celery Lettuce and chicory Onion, garlic Carrot	Soft rot covered with gray mold	Infection before or after harvest through damaged or senescent tissues, by contact with decaying vegetables; direct penetration of cuticle in the case of "ghost spots" of tomatoes	3, 79, 203, 204, 466, 514
Ceratocystis fimbriata	Sweet potato	Firm rot of dark color	Contamination from infected mother plant and wound infection	466

continues

Table 25-4 Continued

Microorganisms	*Vegetable*	*Symptoms*	*Mode and Stage of Contamination*	*Reference*
Chalara spp.	Carrot	Black patches on the roots	Infection of wounds at harvest	411, 466
Choanephora cucurbitarum	Cucurbits	Soft rot	Infection through decaying flowers—Warm climate	466
Cladosporium spp.	Cucurbits Tomato, pepper, aubergine	Small dark superficial lesions (scab)	Direct penetration or penetration through wounds of injured skin—Mostly initiated before harvest	466
Colletotrichum gloeosporioides, C. coccodes, C. capsici, Colletotrichum spp.	Cucurbits Tomato, pepper, aubergine Green beans Yam	Anthracnose, lesion on the skin	Infection before harvest, direct penetration—Development of decay during maturation of the fruits	79, 217, 334, 352, 466
Colletotrichum circinans	Onion	Smudgy spots on the bulb	Direct penetration of the scale	466
Fusarium oxysporum, F. solani, F. moniliforme, and Fusarium spp.	Cucurbits Tomato, pepper, aubergine Asparagus Onion, garlic Potato Cassava Taro Sweet potato Yam	Lesion covered with white to pinkish mold, dry rot on potatoes, cassava, taros. Symptoms may vary with the species	Infection before harvest from the mother plant or wound infection	3, 41, 79, 466
Geotrichum candidum				
	Cucurbits Tomato Carrot	Soft rot with a sour odor	Wound infection before or after harvest	79, 466
Geotrichum candidum				
	Potato	Brown lesion with a rubbery texture	Infection before harvest of immature tubers	466
Helminthosporium solani				
	Potato	Brown patches on the tubers	Infection before harvest. Dissemination may occur during storage	466
Macrophomina phaseolina				
	Cucurbits	Dry rot, eventually black	Infection by contact with soil—Warm climate	466
Microdochium panattonianum	Potato			
	Lettuce and chicory	Spots and yellow dry lesions	Direct penetration or infection via stomata of leaves before harvest	466

continues

Table 25-4 Continued

Microorganisms	*Vegetable*	*Symptoms*	*Mode and Stage of Contamination*	*Reference*
Mucor spp.	Aubergine, tomato, pepper	Soft, very wet rot	Infection before harvest, development of decay during storage	79, 365, 418, 466
Mycocentrospora acerina	Celery Carrot	Dark lesions	Contamination in the field, mostly via wounds, but decay development during storage as tissues of vegetables lose their resistance	300, 466
Mycosphaerella brassicola	Brassica	Dark lesions	Direct penetration before harvest and infection through wounds at harvest	466
Myrothecium rodidum	Tomato, pepper, aubergine	Circular, firm, sunken lesions	Wound infection—Decay mostly on ripe fruits	466
Penicillium spp.	Cucurbits Onion, garlic Yam	Lesion or rot covered with blue-green spores	Infection through wounds or injuries, at or after harvest	466
Peronospora parasitica	Brassica	Gray to dark lesions with sparse mold	Infection in the field. Dissemination by contact during storage	466
Phoma spp.	Cucurbits Tomatoes, pepper, aubergine Brassica Celery Potato	Sunken brown to black lesions with black pycnidia, agent of gangrene of potatoes	Infection before or at harvest through wounds, or from infected mother plant	466
Phomopsis spp.	Tomatoes, pepper, aubergine	Sunken brown lesions	Direct penetration (aubergine) or infection of injured tissues before harvest. Soil inoculum	466
Pythium spp.	Cucurbits Green beans and peas Potato Taro	Soft rot	Before harvest, direct penetration by stomatae or injuries from soil inoculum, and infection from the mother plant. Dissemination after harvest by contact with decaying vegetables	466
	Carrot	Cavity spot		466, 220
Phytophthora spp.	Cucurbits Tomato, pepper, aubergine Green beans and peas Asparagus, carrot	Lesions covered with white mold	Direct penetration before harvest from soil inoculum and infection after harvest by contact with decaying vegetables	300, 466

continues

Table 25-4 Continued

Microorganisms	*Vegetable*	*Symptoms*	*Mode and Stage of Contamination*	*Reference*
Phytophthora erythroseptica	Potato	Firm rot with a pink to dark color	Infection before harvest from the mother plant, dissemination after harvest by contact with decaying tubers	466
Phytophthora infestans	Tomato Potato	Dry and firm rot	Infection before or at harvest. Direct penetration or penetration through natural openings and wounds	79
Phytophthora porri	Brassica Onion, leek	Firm rot	Infection from the mother plant and at harvest by wounds	466
Polyscytalum pustulans	Potato	Dark spots on the skin	Infection before harvest from the mother plant, infection at or after harvest by wounds	466
Rhizoctonia carotae	Carrot	Sunken craters with mold	Infection before or after harvest	466
Rhizoctonia solani	Cucurbits Tomato Green beans and peas Brassica Lettuce and chicory Potato	Firm sunken cankers, black scurf on potato tubers	Infection before harvest by contact with soil or after harvest by contact with decaying vegetables	3
Rhizopus stolonifer and *Rhizopus oryzae*	Cucurbits Tomato, pepper, aubergine Green beans and peas Sweet potato	Soft, very wet rot	Infection at or after harvest by wound or by contact with infected vegetables	79
Sclerotinia sclerotiorum and *S. minor*	Cucurbits Tomato, pepper, aubergine Green beans and peas Brassica Artichoke Celery Lettuce and chicory Carrot	Soft rot with white mold	Infection before harvest from senescent tissues and after harvest by contact with infected vegetables	3, 466
Sclerotium cepivorum	Onion	Soft rot with white mold	Infection from soil before harvest	466

continues

Table 25-4 Continued

Microorganisms	*Vegetable*	*Symptoms*	*Mode and Stage of Contamination*	*Reference*
Sclerotium rolfsii	Cucurbits Tomato, pepper, aubergine Green beans and peas Onion, garlic Carrot Potato Taro Sweet potato	Soft rot	Infection before harvest from soil, and after harvest by contact with decaying vegetables	466
Stemphylium herbarum	Tomato Lettuce	Dark and firm lesion	Infection before harvest by wounds or cracks and decay development during storage on mature tomatoes. On lettuce, infection by stomatae	79, 466
Trichothecium roseum	Cucurbits Tomato, pepper, aubergine	Firm lesions covered with pink spores	Penetration by wounds at harvest and infection from stem or flower scars	466
Yeasts				
Kluyveromyces marxianus	Onion	Soft rot of bulb		260
Bacteria				
Bacillus polymyxa and *Bacillus* spp.	Cucurbits Garlic Potato Pepper Tomato	Soft rot	Presumably wound infection under warm conditions	41, 176, 210, 315, 466
Clostridium puniceum and Clostridium spp.	Potato	Soft rot	Infection via lenticels	315, 321
Erwinia carotovora and *Erwinia* spp.	Cucurbits Tomato, pepper, aubergine Brassicae Asparagus Celery Lettuce and chicory Onion Carrot Potato	Extensive soft rot	Infection by wounds, scars, and lenticels before or after harvest, and by contact with decaying vegetable	193, 315, 466, 518

continues

Table 25-4 Continued

Microorganisms	*Vegetable*	*Symptoms*	*Mode and Stage of Contamination*	*Reference*
Lactobacillus spp.	Onion	Sour rot of bulb scales		315, 466
Leuconostoc mesenteroides	Tomato	Firm, sour rot	Infection via wounds	135
Pseudomonas aeruginosa	Onion, garlic	Dry rot of bulb scales		315, 466
Pseudomonas cepacia, Pseudomonas gladioli pv *alliicola*	Onion, garlic	Soft, slimy rot of bulb scales	Wound infection	315, 466
Pseudomonas viridiflava, P. fluorescens, Xanthomonas campestris, Cytophaga spp.	Cucurbits Tomato, pepper, aubergine Brassicae Asparagus Celery Lettuce and chicory Carrot	Soft rot	Infection via wounds, scars, before or after harvest, and by contact with decaying vegetables. *Xanthomonas campestris* may infect leaves directly before harvest	315, 466
Pseudomonas syringae various pathovars specific for the genus of vegetables	Cucurbits Green beans and peas	Water-soaked spots, which may extend to soft rot	Field disease, but may develop during storage	315, 466

veloping countries if storage facilities and hygienic standards for foods are not adequate.

The presence of pathogenic microorganisms on vegetables can be a cause of human diseases. Vegetables were probably the source of *P. aeruginosa* responsible for infections in hospitals in Brazil: some of the strains isolated from vegetables in hospital kitchens and strains from patients suffering blood, urine, or wound infections had the same serotypes and pyocin types,[138] although cross-contamination of vegetables from the hospital environment cannot be excluded. Vegetables are a minor cause of foodborne incidents of microbial origin, and were incriminated in only 4.8% of cases in Canada from 1975 to 1984,[501] or in only 2% of cases with a known vehicle in the US from 1973 to 1987.[59] However, processed vegetables are the main source of botulism in the US[59] and the number of foodborne diseases associated with fresh fruits and vegetables is increasing.[23] Consumption of contaminated vegetables, either fresh or processed, was suspected to be the origin of cases and outbreaks caused by the main foodborne pathogens (Table 25-5). Vegetables offer, therefore, a sufficient potential for development and survival of foodborne pathogens to transmit human diseases. In some cases, the origin of contamination with the pathogenic organism was traced (Table 25-5), illustrating the need for hygienic practices during cultivation and processing. This is supported by cases of *Salmonella*,[10,123] *E. coli* O157:H7,[70] and virus[415] transmitted by fresh or minimally processed fruits.

25.2.3 Origin of Microorganisms Found on Vegetables

During emergence from the seed, plants become contaminated with large numbers of microorganisms. The first leaves of green endive (*Cichorium endivia*) carried 10^3 cfu cm^{-2}.[369] For sprouts grown in sprouting chambers, the seed is probably the main source of microorganisms. Contamination of sprouts with *B. cereus*[227,403] and *Salmonella* spp.[386,402] caused foodborne disease (Table 25-5), and the bacteria were isolated from the ungerminated seed. On witloof chicory forced under soil cover or under hydroponic systems, part of the microflora of leaves was similar to that of the roots used to grow chicory heads.[507] Seeds are also a source of some post-harvest diseases of vegetables, for example, *Colletotrichum* on pepper.[334] Transmission of post-harvest diseases is more frequent in the case of vegetative seeds, eg, *Botrytis* spp. is the cause of onion bulb neck rot,[344] *Erwinia* spp. is the cause of potato tuber soft rot,[395] and *Phoma* spp. is the cause of gangrene of potato tubers.[310] In such cases, seed transmission is of critical importance because the microorganisms are not common in soil or in the environment.

Some of the predominant microorganisms of vegetables have also been found in the field environment including surface water, irrigation water, soil, and neighboring plants. Contamination with microorganisms from the environment is now recognized as an important feature in the microbial population of cultivated plants.[307] Pectate-degrading bacteria and fluorescent pseudomonads were abundant both on vegetables at harvest and in irrigation water.[382,426] *Rahnella aquatilis*, a member of the Enterobacteriaceae commonly isolated from vegetables, is primarily a bacterium from the aquatic environment.[201] Bacterial spores and resistant forms survive in soil for long periods; the soil is thus a source of contamination for plants with spoilage microorganisms such as soft rot bacilli or clostridia,[315] *Sclerotinia* spp.,[466] *Sclerotium rolfsii*,[409] and *Rhizoctonia solani*.[466] *Sclerotinia* spp.[81,466] attack many weeds, which can be a source of contamination for vegetables. Potential human pathogens such as *P. aeruginosa*, pathogenic bacilli, or clostridia are usually present in soil.[218,289,316,385] *Listeria monocytogenes* was isolated from a significant proportion of samples of soil, water, and plants analyzed in cultivated or uncultivated areas.[133,158,529,530] Thermotolerant *Campylobacter* were isolated from 20% to 55% of samples of river and pond waters in England and North America.[390]

The presence of many foodborne pathogens on vegetables at harvest is frequently assumed to result from a contamination by polluted water or organic fertilizer.[23,98,163,390,425] This is illustrated by a few documented outbreaks of foodborne diseases presented in Table 25-5. *Listeria monocytogenes* was detected in between 100% and 44.6% of samples of sewage or sewage sludge,[8,328,449,523] with numbers up to 50 g^{-1}. Ten percent of samples of alfalfa grown in soil fertilized with sewage sludge cake bore 3 to 5 *L. monocytogenes* g^{-1} at harvest.[8] Wastewater and excreta are widely used in agriculture as a source of fertilizer and irrigation in Asian, African, and Latin American countries,[338] which stresses the importance of this source of contamination.

Domestic animals are a source of bacteria such as *Salmonella*, thermotolerant *Campylobacter*, and *Yersinia* spp., and may also disseminate organisms that cause post-harvest spoilage of vegetables, such as sclerotia of *Sclerotium* spp. and *Sclerotinia* spp., which may be ingested with plant material.[466] Wild

Table 25-5 Examples of Foodborne Diseases and Outbreaks Linked to the Consumption of Vegetables

Outbreak (Country/Date)	*Pathogen*	*Number of Cases*	*Number of Deaths*	*Suspected Vegetable*	*Reference*
Fresh and minimally processed vegetables					
US/1973	*Bacillus cereus*[a]	4	0	Salad sprouts	403
US/1987	*Clostridium botulinum* (type A)[a]	4	0	Cabbage in dressed salad[b]	469
US/1995	*Escherichia coli* O157:H7	NS	NS	Lettuce	25
US/1993	*E. coli* enterotoxigenic (O6)	168	0	Shredded carrots	363
US/1979	*Listeria monocytogenes* 4b	20	5	Raw tomatoes, lettuce, and celery[c]	236
Canada/1981	*L. monocytogenes* 4b[a]	41	17	Vegetable mix for coleslaw[d]	446
UK/1988	*Salmonella* St-Paul[a]	143	0	Bean sprouts	386
UK/1988	*Salmonella* Virchow[a]	7	0	Bean sprouts	386
Sweden/1988	*Salmonella* St-Paul[a]	148	0	Bean sprouts	9
Finland and Sweden/1994	*Salmonella bovismorbificans*[a]	492	0	Bean sprouts	402
US/1986	*Shigella sonnei*	347	0	Shredded lettuce[e]	142
Norway/1994	*S. sonnei*	110	0	Imported iceberg lettuce	271
Israel/1970	*Vibrio cholera*	176	NS	Various raw vegetables[f]	460
US/1971	Hepatitis virus	129	0	Raw watercress[g]	243
US/1988	Hepatitis A virus	202	0	Lettuce	429
US/1995	*Cryptosporidium*	15	0	Mixed salad with celery	71
US/1989	*Giardia*	21	0	Raw lettuce, onions, and tomatoes	125
Processed vegetables					
US (New Mexico)/1978	*Clostridium botulinum* (type A)[a]	32	0	Potato and bean salads	121
US (Colorado)/1978	*C. botulinum* (type A)	7	0	Baked potatoes	451
US/1983	*C. botulinum* (type A)[a]	28	1	Sauteed onion left under a layer of set margarine	327
Canada/1985	*C. botulinum* (type B)	39	0	Garlic chopped in oil	484
Canada/1987	*C. botulinum* (type A)[a]	11	0	Bottled in-house mushrooms	124
Italy/1989	*C. botulinum* (type B)	5	0	Black olive	185
Italy/1993	*C. botulinum* (type B)	7	0	Eggplant roasted and bottled in oil	22
US/1994	*C. botulinum* (type A)[a]	23	0	Baked potatoes in aluminium foil	18
Spain/1996	*C. botulinum*	2	0	Home-canned green beans	401
Finland/1989	*L. monocytogenes* 4b	1	0	Salted mushrooms (7.5% sodium chloride)	263
US/1989	*Staphylococcus aureus*[a]	>100	0	Imported canned mushrooms	226

[a] Pathogen and/or toxin detected in the suspected food vehicle.
[b] Toxin production in modified atmosphere packaged cabbage suspected.
[c] Vegetables prepared in hospital kitchen.
[d] Field contamination of cabbage with manure suspected.
[e] Contamination by food handlers suspected.
[f] Contamination by irrigation with untreated wastewater.
[g] Pollution of surface water by septic tanks suspected.
NS = Not stated.

birds may carry *L. monocytogenes*[186] and insects such as *Drosophila melanogaster*, that feed on wounded and decaying fruits and vegetables, can disseminate soft rot *Erwinia* spp.,[315] *Geotrichum candidum*, *Rhizopus* spp., and *Botrytis cinerea*.[103,312]

The presence of microorganisms on harvested vegetables depends on the capacity to survive or to multiply on the plant before harvest. On bean sprouts, the microbial load increased during germination and sprouting from 5–6 log cfu g^{-1} on the seed to 8 log cfu g^{-1},[358,477] but the proportion of enterobacteria increased during sprouting, from less than 0.1% of the total bacteria population in the seeds to 60% in the sprouts. *Bacillus cereus* on bean seeds at 21°C[477] and *Salmonella stanley* on alfalfa seeds at 30°C[255] rapidly multiplied during germination and sprouting, whereas *Staphylococcus* spp. did not multiply and represented a minor part of the microbial population on sprouts.[477] Many post-harvest disease agents colonize the mother plant, produce inoculum, and contaminate the vegetables before harvest (Table 25-4). This is the case with *Botrytis* spp., *Fusarium* spp., and *Sclerotium rolfsii* on various vegetable crops, *Phytophthora* spp. on solanaceous vegetables, *Alternaria* spp. on carrots, and soft rot erwinias on potatoes.[152,466] The plant or its immediate environment may also provide conditions for survival of microorganisms not commonly found in the field. For example, the soft rot bacterium *E. carotovora* survived for a longer period in the rhizosphere of crops than in the soil[315]; pectolytic fluorescent pseudomonads were not found in soil[442] but were abundant in a field where carrots had suffered extensive soft rot[141]; *Listeria monocytogenes* was also suspected to survive better in the rhizosphere of plants than in nonrhizosphere soil.[508] Pathogenic bacteria,[8,170,219,382,432,438,508] viruses,[37,219,288,438,498,521,522] and helminths[431] declined on cultivated plants, but in some studies pathogens survived until harvest.

Numbers of microorganisms may increase during harvesting or handling. The following factors may contribute to this: lack of sanitation facilities for farm workers and poor personal hygiene,[23,207,390] persistence of microorganisms on harvesting equipment,[51,257,473] and multiplication of microorganisms on produce during exposure to warm, moist conditions during harvesting and transport.[455,457] Potato tubers may become contaminated at harvest by *Alternaria alternata* from infected foliage,[168,169] and the use of water to transport potatoes in flumes or for washing may spread contamination with soft rot *Erwinia* spp.[48,53,452] Decaying products in storerooms are another important source of contamination (Table 25-4).

25.2.4 Localization of Microorganisms on Vegetables

On lettuce, chicory, cabbage, or bulbs where leaves are tightly arranged in a head, a decreasing gradient of microbial contamination was observed from outer to inner leaves. Numbers of mesophilic bacteria on outer leaves of lettuces could be 1 to 3 log cycles higher than on inner leaves.[4,280] On white cabbage, leaves are very tightly closed and the number of microorganisms decreased by 4 log cycles from the outer to the central leaves,[281] and only a few microorganisms could be isolated from the core of the cabbage heads.[202] Similar observations were made for witloof chicory[507] and fennel bulbs.[179] On green endive (*Cichorium endivia*), which have looser heads than iceberg lettuce, differences between inner and outer leaves were of 1 log cycle or less.[369] Localization of microorganisms on the surface of vegetables was investigated in a few cases. On green endive leaves[216] and spinach,[35] bacteria were not dispersed over the cuticle, but appeared as microcolonies embedded in a fibrillar matrix, and preferentially located near stomata, in cuticular cracks, or around epidermal hairs, which could be an adaptation of bacteria to the climatic conditions in the field.[157] This is consistent with the observations that bacteria removed from chicory leaf surfaces by a water rinse appeared as microcolonies and not as single cells.[370] However, other observations found individual bacterial cells scattered on the surface of lettuce leaves.[4] On potato tubers, pectolytic *Erwinia* have regularly been isolated from lenticels[210,395] where they survive for longer periods than on the surface of tubers.[315,395] The attachment of phytopathogenic bacteria to plant surfaces was recently reviewed[428] but there is little information on adhesion of saprophytic bacteria on vegetables. Adhesion of conidia of *B. cinerea* to tomato cuticle was recently characterized as a hydrophobic interaction without specific attachment structures, germination increasing adhesion with the secretion of a sheath of base-soluble material.[162]

Fungal agents of storage diseases may have colonized a few cells or a limited area of vegetable tissue at harvest without causing visible spoilage. This is referred to as "quiescent" or "latent infection."[256,408,493] Some fungi such as *B. cinerea*, the cause of ghost spots on tomato fruits, and *Colletotrichum* sp., the agent of anthracnose on pepper, upon contact with the surface of the epidermis may form a specific organ called an appressorium, which permits strong adhesion and penetration of the cuticular layer. More generally, fungi may be present in senescent plant tissues that remain on the vegetable after harvest and trimming, for example, *Sclerotinia* spp., *Botrytis* spp., and *Alternaria* spp. in the basal part of leaves of onion bulbs or of carrot roots, in the calyx of tomatoes, and in the scars left by stem or flowers on many fruit vegetables (Table 25-4). Bacteria, including *Pseudomonas, Xanthomonas, Erwinia,* and, less frequently, *Corynebacteriaceae* have also been isolated from internal tissues of sound cucumber, tomato, green bean, garden pea,[441] potato,[210] and Chinese cabbage.[315] In tomatoes, bacteria were more frequent in the core underlying the stem scar whereas in cucumber they were mostly isolated from the periphery,[441] presumably reflecting a penetration via stem scar or via stomata, respectively. In potato, pectolytic *Erwinia* were isolated from vascular and not from cortical regions of the tubers,[210] presumably transmitted from the rotting mother tubers.

25.3 THE MICROBIOLOGY OF FRESH VEGETABLES DURING STORAGE

25.3.1 Fate of Epiphytic Microorganisms

Numbers of microorganisms on vegetables usually increase during storage, even under refrigeration, which is consistent with the high proportion of psychrotrophs found by many investigators. Aerobic mesophilic bacteria increased from 5.1 log cfu g^{-1} to 6.3 log cfu g^{-1} in 15 days at 2.8°C on iceberg lettuce[280]; from 5.3 log cfu g^{-1} to 7.9 log cfu g^{-1} in 3 weeks at 10°C on white cabbage[281]; from 6.5 to 9 cfu g^{-1} in 6 weeks at 1°C on broccoli[87]; and from 4.8 to 5.8 in 4 weeks at 13°C on tomatoes.[86] At 1° to 2°C, Geeson[202] noted only small changes in the numbers of bacteria, the predominant microorganisms on white cabbage, but found an increase in the numbers of *Botrytis cinerea,* an important spoilage agent of cabbage during storage. Bacterial counts increased only on cabbage drenched with fungicides before storage, presumably because of the presence of free water. All these reports concerned vegetables that did not show visible spoilage.

25.3.2 Development of Microbial Spoilage

Sound vegetables regularly harbor spoilage microorganisms, but infection and development of decay are the results of complex interactions between virulence factors of the spoilage agent and defense mechanisms of the vegetable tissues. The first step is penetration of the vegetable tissues. Upon penetration, microorganisms may invade the host tissues or may be kept in check by the defense mechanisms of the vegetable. In these latter cases the infection is either stopped or the microorganims may remain quiescent until maturation, ageing, or any form of scenescence that reduces the defense mechanisms of the vegetable and permits the development of infection.[256,408,493]

Entry of Microorganisms

The surface of vegetables shows a large diversity in structure and composition; the epidermis is covered by a cuticular layer in aerial organs (leaves, stem, flowers, and fruits) or periderms for roots or tubers. Stomata, lenticels, hair sockets, and scars from detached organs represent natural ways of entry for microorganisms. Cracks in the vegetable surface may occur in certain growing conditions,[174,394] and post-harvest handling may cause injuries and bruising. Some microorganisms can penetrate through the cuticular layer (Table 25-4), eg, *B. cinerea* on tomato fruits,[422,440] *B. cinerea* on cucumber fruits,[178] *Colletotrichum* spp. on tomato and bell pepper,[6,493] and *Colletotrichum circinans* on onion bulbs.[466] Most microorganisms, however, penetrate into vegetables through injured tissues. Spores of *Rhizopus stolonifer* were unable to infect intact tomato fruits[42] but caused a rapid infection on mechanically wounded fruits or on cold-injured fruits. A black rot agent of carrot (*Chalara elegans*) did not cause lesions on unwounded roots,[411] and most pathogens of carrots are unable to penetrate undamaged roots.[303] Under some conditions, scars or lenticels allow invasion of vegetables by soft rot *Erwinia* spp. Hydrostatic pressure caused infiltration of lenticels of potato tubers[53] and infiltration of stem scar tissue of tomato fruits.[48–50] The presence of a film of free water on the surface of potato tubers resulted in bacterial invasion via lenticels and wounds,[320] and infection of inoculated lenticels was observed only when tubers had been incubated at high RH (>80%). Penetration may also occur *via* dead tissues colonized before harvest. *Sclerotium rolfsii* or *Sclerotinia* spp. usually invade the foliage of the carrot plant in the field and then progress to the crown region of the root.[409,466] Similarly *B. cinerea* frequently infects tomato fruits after colonization of the calyx.[152] Fungicide treatment of the calyx reduced incidence of *B. cinerea* and *A. alternata* on eggplant and bell pepper.[181]

Nature of Inoculum

The inoculum of fungal agents of post-harvest diseases of vegetables may be spores or conidia, mycelium, and sclerotia. Mycelium is usually infectious, but spores and sclerotia need to germinate in order to cause infection. Germination of conidia from *Colletotrichum* spp. is influenced by compounds leaching from the surface of the vegetable[493] and by ethylene production.[190] Germination of sclerotia of *Sclerotium rolfsii* and infection of vegetables depend on relative humidity and the presence of certain volatiles.[409] Germination of spores of *Botrytis cinerea* on cabbage leaves from a range of cultivars was positively correlated with the incidence of *Botrytis* rot on cabbage heads during storage.[143] Germination may depend on the availability of nutrients: *Botrytis cinerea,* normally a wound pathogen of carrot, can infect undamaged roots in the presence of nutrients[303]; on onion, conidia of *B. cinerea* suspended in water were unable to cause infection, whereas in the presence of nutrients conidia showed a higher germination rate, longer germ tubes and caused infection[131]; in contrast, conidia of *Botrytis squamosa* on onion were not dependent on nutrients. Mycelium of *Rhizopus stolonifer* was infectious on wounds of sweet potato, but neither dormant spores nor germinated spores were able to invade wounded sweet potato tissues unless macerated sweet potato tissues, and therefore nutrients, were present in the wound.[483] In practical conditions, mycelium produced from infected tissues by some fungi such as *B. cinerea, R. stolonifer, S. rolfsii, Sclerotinia* spp., and *Rhizoctonia carotae* may infect adjacent vegetables during storage, causing a nest of rot and significantly increasing losses during storage (Table 25-4). Although spores of *R. stolonifer* can infect only wounded or injured tomato fruits, undamaged fruits are readily infected by contact with decaying fruits.[42] Senescent leaves on onion bulbs, decaying petioles on carrot roots, or sepals on tomato fruits are important in the infection of vegetables by *B. cinerea* by providing a nutritive base to the fungus.

Virulence Factors of Spoilage Organisms

For microorganisms penetrating directly into the vegetable tissues through the cuticle, production of cutinolytic enzymes[284] is the first step of infection. Among organisms that cause post-harvest diseases of vegetables, *B. cinerea,*[440] *B. squamosa,*[39] *Colletotrichum gloeosporioides,*[284] and *Sclerotium rolfsii*[39] produce cutinase whereas *Rhizopus stolonifer* failed to do so,[378] although the mycelium of the fungus can attack undamaged tomato fruits by contact with infected fruits.[42] Production of biosurfactant was an important factor in the infection of undamaged broccoli heads by pectolytic fluorescent pseudomonads.[233] Pectolytic pseudomonads that did not produce biosurfactants could only infect wounded tissues but could invade undamaged broccoli in the presence of biosurfactant-producing strains. The role of biosurfactant was probably to increase contact between the bacteria and the waxy surface of broccoli.

Many diseases are characterized by maceration of the vegetable tissues (ie, soft rot symptoms) as a result of enzymic degradation of cell wall polysaccharides. Pectin-degrading enzymes[400] play a key role in the development of infection. They are produced early during the germination of fungal spores, and may even be present in the spores before germination.[516] Purified pectin-degrading enzymes were able to cause maceration of vegetable tissues and cell death, without involvement of other enzymes or toxic factors.[55,504] However, microbial pathogens frequently produce several types of pectin-degrading enzymes, which do not always have the same role. Pectin trans-eliminase from *Fusarium oxysporum* f. sp. *cepae* caused maceration of onion bulb whereas the polygalacturonase did not.[238,239] Mutants of the soft rot bacterium *Erwinia chrysanthemi* that did not produce pectin methylesterase or one isoenzyme of pectate lyase had a reduced ability to macerate potato tubers, whereas the other pectate lyase isoenzymes did not seem to be necessary for tissue maceration.[45] The aggressiveness of isolates of *Mycocentrospora acerina* on carrots was related to their production of pectinase and glucanase.[298] In contrast, no production of pectin-degrading enzymes by *C. elegans,* the agent of black rot of carrots, was found.[411]

Oxalic acid is produced by *Sclerotinia* spp.,[330] *Sclerotium rolfsii,*[57,409] *Penicillium oxalicum* on yam,[247] and *Rhizoctonia carotae.*[410] It may contribute to virulence, acting synergistically with pectin-degrading enzymes by sequestering calcium and thereby weakening the middle lamella of the cell wall, or by lowering the pH to a level more favorable to the activity of fungal pectinases. Oxalic acid itself may be toxic to the host tissues and may interfere with the defense mechanisms of the host tissues by inhibiting polyphenol oxidase.[189,330]

Defense Mechanisms of Vegetables

Plant tissues have both preformed barriers against infection and barriers induced in response to infection. Storing vegetables in conditions favorable to the development of such barriers is an important means of reducing decay. Cuticle, epiderm, or periderm tissues are the first preformed barriers against microorganisms. Removing waxes from cuticle increased infection of pepper fruits by *Colletotrichum* *capsici* or *Colletotrichum gloeosporioides*[330] and of cabbage by *B. cinerea*.[448] The lower incidence of *Rhizopus stolonifer* infection on green tomato fruits than on mature ones can be explained by a higher resistance of the epidermis of green fruits. Once injured or wounded, fruits from both stages of development were equally susceptible.[42] The composition of the cell walls may explain in part the resistance of some vegetables. Resistance could be linked to a higher proportion of protopectin (ie, nonsoluble pectin) in the cell wall of carrot,[299] a higher degree of methylation of the pectin,[45,351] or a higher level of calcium in potato tubers.[137,349,350] In these examples, cell walls were presumably more resistant to enzymic degradation. Protein inhibitors of microbial pectinase were detected in tomato fruits[94] and in beet root tissues.[99] The purified inhibitor protected beet root tissues from pectinase of *R. solani* and *Phoma betae* and was in higher concentration in resistant roots.

Resistance of vegetables to infection may be due to preformed antimicrobial compounds. The presence of the phenolic compounds catechol and protocatechuic acid inhibits infection of outer scales of colored onion bulbs by *Colletotrichum circinans*, the agent of smudge disease.[335,466] The periderm and pericyclic tissues of carrot roots, unlike the xylem and to a lesser extent phloem, are very resistant to infection. This was attributed to the high concentration of the polyacetylenic compound falcarindiol.[199] The concentration of falcarindiol in xylem was only 2% of that found in periderm and pericyclic tissues where the concentration (93 $\mu g\ g^{-1}$ fresh weight) was sufficient to inhibit germination of spores of *M. acerina* (ED_{50} of 31.8 $\mu g\ ml^{-1}$). Tomatine could explain the resistance of green tomato fruits to *B. cinerea*, although the fungus can detoxify tomatine.[408,515]

In response to infection by microorganisms, or to various chemical or physical agents (eg, wounds, radiations, heat or cold shocks, ethylene, biotic elicitors), plant tissues may form defensive barriers.[159] These may consist of the synthesis of small molecules, belonging to various chemical groups (phenolics, terpenoids, alkaloids), with antimicrobial properties, named phytoalexins[335] or in the deposition of callose, suberin, or lignin on the cell wall. Phytoalexins play a role in the resistance of freshly harvested carrots[126,214,225] or immature pepper fruit[7] to fungal pathogens. However, accumulation of phytoalexins by potato tubers from different cultivars was not related to resistance to *Erwinia carotovora* var *atroseptica*.[62,63,326] Wounds or infection generally trigger the deposition of lignin on the cell wall or the formation of a suberized layer that can prevent the development of infection,[421] as in the case of potato tuber and soft rot bacteria.[51] In carrot roots, however, wound healing is crucial for resistance to spoilage agents but was attributed more to accumulation of antifungal compounds than to suberization.[200] Other mechanisms involved in resistance of plants, such as lipoxygenase,[461] chitinase,[439] or accumulation of hydroxyproline-rich proteins,[433] are triggered by infection in some vegetables but there is no indication of their role in resistance to spoilage.

In many vegetables the ability to resist spoilage microorganisms decreases during storage. In carrot roots, latent infections with some fungi became progressive after a few months, presumably because of a decline in the ability to accumulate antifungal compounds.[303]

25.3.3 Fate of Foodborne Pathogens

The survival or growth of microorganisms pathogenic to humans on the surface of unprocessed raw vegetables depends on the presence of free moisture, relative humidity, temperature, and exposure to sunlight. Bacteria can grow on vegetables during storage whenever temperatures are favorable (Table 25-6). In some studies, high numbers were reached before the onset of spoilage. Viruses survive for several days on the surface of vegetables under refrigeration and high humidity (Table 25-7), but decline steadily under dry conditions. Microorganisms that are potentially pathogenic to humans and contaminate vegetables during cultivation or at harvest would likely survive, or even increase in numbers, during storage before consumption or processing.

25.4 THE EFFECT OF MINIMAL PROCESSING ON THE MICROBIOLOGY OF VEGETABLES

25.4.1 Treatments Used in Minimal Processing

In this chapter included as minimal processing is any treatment of the vegetables that produces a ready-to-eat or ready-to-use product, but without lethal effect on the vegetable tissues. This excludes heat treatment, freezing, drying, fermentation, or the addition of acid dressings. Minimally processed vegetables are usually trimmed, peeled, cut if necessary, washed, and sometimes disinfected, and frequently packaged in polymeric films. In this latter case, respiration of the vegetable in a sealed package rapidly reduces the concentration of oxygen and increases that of carbon dioxide.[509] The production of minimally processed vegetables started about 30

Table 25-6 Fate of Foodborne Pathogenic Bacteria on Raw Vegetables during Storage

Bacteria	*Vegetables*	*Initial Number (log cfu g^{-1})*[a]	*Temperature (°C)*	*Growth Rate (log d^{-1})*	*Maximum Number or Number Reached at Spoilage (log cfu g^{-1})*[a]	*Reference*
Aeromonas spp.	Various vegetables	<2 to 3 (natural contamination)	5	0.15 to 0.5	NS	104
Aeromonas hydrophila	Asparagus, broccoli, and cauliflower[b]	3 to 5	15	1.2 to 1.7	8 to 9	66
			4	0.2 to 0.3	6 to 8	
Listeria monocytogenes	Tomato[b]	3.7	21	1	5	74
			10	0 to 0.2	5	
L. monocytogenes	Asparagus, broccoli, and cauliflower[b]	3 to 5	15	1	7 to 9	67
			4	0 to 0.15	3 to 6	
L. monocytogenes	Various vegetables[b]	5.7	4	−0.02 to 0.02	NS	90
Salmonella montevideo	Tomato[b]	1.7 log cfu cm^{-2}	10	No growth		542
			20	0.8	4 log cfu cm^{-2}	
			30	2.5	5 log cfu cm^{-2}	

[a] Unless specified otherwise.

[b] High humidity maintained by humidification or by packages.

NS = Not specified.

years ago in the US and was intended mainly for the catering industry. The production has increased rapidly since 1990 because of the development of a retail market.[447] In Europe, production for the retail market started in the early 1980s and grew rapidly. A wide range of vegetables are now minimally processed, including green salads, mushrooms, carrots, celeriac, sprouts, cabbage, onion, broccoli and cauliflower, and vegetable fruits.[12,447]

25.4.2 Microbial Contamination of Minimally Processed Vegetables

Spoilage and Saprophytic Microorganisms

The numbers of microorganisms found on unstored minimally processed vegetables are of the same order of magnitude as those reported for raw vegetables and range from 10^3 cfu g^{-1} on shredded iceberg lettuce to 10^6 cfu g^{-1} on shredded carrots[337] (Table 25-8), with numbers reaching 10^7 cfu g^{-1} for bean sprouts.[510]

The predominant microbial species identified in minimally processed vegetables are those found in whole, unprocessed vegetables.[377] *Pseudomonas, Enterobacter, Erwinia,* and *Rahnella* usually prevail over other genera.[35,47,93,100,116,198,206,280,282,329,336,339,381] Among pseudomonads, most isolates fell into the species *P. fluorescens* and less frequently *P. putida* and *P. chlororaphis.*[254] The most frequently identified Enterobacteriaceae are *Enterobacter agglomerans, Erwinia herbicola,* and *Rahnella aquatilis. Enterobacter intermedium* and *E. cloacae* are less frequently reported. Species of the genus *Serratia, Xanthomonas,* and *Flavobacterium* seem to be less common. Psychrotrophs are an important component of the microflora,[47,198] as discussed for raw vegetables. Populations of pectinolytic microorganisms were determined by various authors as potential spoilage agents, and found to represent 10% to 20% of isolates among mesophilic bacteria from shredded lettuce,[329] or 20% to 60% of fluorescent pseudomonads in many samples of shredded carrots and shredded green endives.[116,381] Populations of pectinolytic bacteria in various mixed salads were estimated as 1% of total mesophilic bacteria.[93] The major pectinolytic species found in minimally processed vegetables[377] were various biovars of *Pseudomonas fluorescens, P. paucimobilis, P. viridi-*

Table 25-7 Fate of Foodborne Pathogenic Viruses on Raw Vegetables during Storage

Viruses	*Vegetables*	*Conditions*	*Temperatures (°C)*	*Incubation Time (d)*	*Survival (%)*	*Reference*
Coxsackie virus B5	Lettuce	Saturated humidity	4	7	91	286
		Low humidity	4	7	36	
		Dry conditions	4	7	<0.8	
Coxsackie virus B5	Celery	Saturated humidity	4	5	100	287
		Dry conditions	4	1	25	
		Idem	4	5	<1	
Poliovirus	Celery	High humidity[a]	4	40	20	521
		Idem	4	76	1	
	Spinach	Idem	4	21	26	
		Idem	4	55	1	
Enteroviruses	Tomato	NS	3 to 8	10	10	37
			18 to 21	10	1 to 10	

[a] Authors did not specify how high humidity was maintained.
NS = not specified.

flava, *P. luteola, Xanthomonas maltophilia, Flavobacterium* spp., *Cytophaga* spp., *Vibrio fluvialis*, and some pectinolytic fungi and yeasts (*Mucor* spp., *Sclerotinia* spp., *Trichosporon* spp.). The main difference between the microflora of unprocessed and minimally processed vegetables is the comparatively high proportion of lactic acid bacteria and yeasts found in some minimally processed products (Table 25-8). In some products, atmospheres in packages promoted growth of lactic acid bacteria.[100,115,117] Predominant lactic acid bacteria in minimally processed vegetables were *Leuconostoc* spp.,[100,150,198] and more particularly *L. mesenteroides*,[116] in accordance with the results presented above for unprocessed vegetables. Numbers of yeasts were 3 to 5 log cycles lower than those of mesophilic bacteria for minimally processed lettuce,[280] endive salads,[339] or mixed vegetables for soup,[337] but could reach 10^7–10^8 cfu g^{-1} in shredded carrots.[34] Yeasts identified from minimally processed vegetables fall into many genera, including *Candida, Cryptococcus, Rhodotorula, Trichosporon, Pichia, Torulaspora*, and *Saccharomyces*.[34,280,329,339]

Table 25-8 Numbers (log cfu g^{-1}) of Microorganisms Found in Minimally Processed Fresh Vegetables

Product	*Mesophilic Aerobic Microflora*	*Lactic Acid Bacteria*	*Yeast and Molds*	*Storage Time (d)*	*Storage Temperature (°C)*	*Atmosphere in Packs $\%O_2/\%CO_2$*	*Reference*
Mixed vegetables	7.7 to 8.4	5.6 to 6	4 to 5	Sell by date +1	7	17/4	93
Mixed vegetables	8.6 to 9	8.4 to 8.9	6.7 to 7.2	Sell by date +1	7	0 to 17/ 6 to 16	93
Various shredded vegetables	4 to 7	ND to 3	ND to 4	Unstored			198
Shredded carrots	6 to 7	3 to 4	ND	Unstored			116
Shredded carrots	8 to 9	4 to 8[a]	4 to 7	14	10	1.5/10 to 30	116
Mixed vegetables	4 to 8	3 to 8	2 to 6	NS	NS	NS	234
Mixed vegetables	8	5.5 to 6.3	4 to 4.2	Unstored			337
Mixed vegetables	8.3	7 to 7.8	3.3 to 5	7	4	NS/10	337

[a] Samples with 8 log cfu g^{-1} lactic acid bacteria were spoiled.
ND = Not detected.
NS = Not specified.

Microorganisms of Public Health Significance

Most of the surveys of foodborne pathogens in minimally processed vegetables (Table 25-9) focused on *L. monocytogenes* because of the general concern about risks of listeriosis with refrigerated ready-to-eat foods and because of the outbreak in Canada linked to the consumption of vegetable mix for coleslaw (Table 25-5).[446] Surveys do not always specify whether products were packaged, and therefore protected from contamination after processing. Products concerned in the studies presented in Table 25-9 were presumably processed in contrasting hygienic conditions, from industries complying with good manufacturing practices to small restaurant kitchens.

In France an incidence of 8.8% was found in 1988,[295] but no positive sample has been reported since 1992 (Table 25-9). In 1996, no *L. monocytogenes* was found in over 605 samples of packaged minimally processed vegetables, whereas the same survey detected 4% to 12% positive samples in other processed vegetables.[399] It is possible that this reduction in contamination for minimally processed vegetables was a result of the application of good manufacturing practices at the end of the 1980s and of the reduction in the number of processors to a few large companies with better hygienic standards. The number of *L. monocytogenes* in positive samples was estimated by the most probable number method to be below 1 g^{-1}.[295] Incidence of *Y. enterocolitica* is usually high (Table 25-9) but all the isolates were considered as nonpathogenic to humans, on the basis of the serotypes and the absence of virulence markers, except in shredded carrots sampled in a restaurant. *Aeromonas hydrophila* was found in a large proportion of samples but it is not known whether these strains had any significance as pathogens. Concern was expressed in the US over the possible development of botulism in products packaged under low oxygen atmosphere,[14] including minimally processed vegetables. In a survey of over 1,118 samples of minimally processed vegetables in the US, *C. botulinum* was isolated from 0.36%.[304] Presence of *Salmonella* spp. and *S. aureus* has not been reported in packaged minimally processed vegetables.

The Effect of Minimal Processing on Numbers of Microorganisms

During minimal processing, the overall contamination of vegetables is reduced by between 1 and 2 log cycles.[198,280,381,502] The removal of the most contaminated part of the vegetables by trimming and peeling (the external leaves of cabbage, lettuces, and green salads, or the periderm of roots) partly accounts for this reduction. The remainder is achieved by the washing procedure, associated in some instances with a chemical disinfection. Nevertheless, some steps in minimal processing could increase the microbial load or could be a source of contamination with foodborne pathogens (Table 25-10).

25.4.3 The Fate of Microorganisms on Minimally Processed Vegetables during Storage

Saprophytic and Spoilage Microorganisms

Packaging and respiration of minimally processed vegetables provide high relative humidity for the growth of microorganisms. However, growth rates are quite variable, even for a single product (Figure 25-1).

In the studies quoted in Figure 25-1, the aerobic mesophilic microflora reached a maximum population of approximately 10^8–10^9 cfu g^{-1} on minimally processed vegetables. Growth rates of lactic acid bacteria and yeasts are more variable than those of aerobic mesophilic bacteria (Figures 25-2 and 25-3) and do not clearly increase with temperature, indicating that growth of these populations may be limited by other factors. High carbon dioxide and low oxygen concentrations in the packages encouraged lactic acid bacteria on shredded carrots,[100,117] on butterhead lettuce,[348] and on shredded endive.[107] The composition of the yeast population of shredded carrots changed during storage[34]: *Cryptococcus albidus*, a nonfermentative species, predominated on unstored carrots and only fermentative *Candida* spp. were isolated after a week at 10°C.

Microorganisms of Public Health Significance

Growth of bacterial foodborne pathogens on minimally processed vegetables is not markedly different from that on raw vegetables (Figures 25-4A and 25-4B). At temperatures between 3° and 10°C, only *L. monocytogenes* and *Aeromonas hydrophila* grew on minimally processed vegetables, with a particularly rapid growth reported for *A. hydrophila*.[31,339] *Escherichia coli, Shigella sonnei*, and *Staphylococcus aureus* survived or decreased in numbers slowly. Above 10°C, most pathogens tested grew, and showed high growth rate as temperature increased, for instance, *S. sonnei* increased 1,000-fold in 12 hours at 22°C on shredded lettuce.[142]

At these temperatures, however, growth may be followed by a rapid death of the pathogen as in the cases of S. *sonnei*[444] and of *L. monocytogenes*[270] on shredded cabbage. *Staphylococcus aureus* is the only bacterial pathogen that showed a poor ability to

Table 25-9 Incidence of Microorganisms of Public Health Significance in Minimally Processed Vegetables

Microorganisms	*Product*	*Country*	*Number of Samples*	*Incidence (%)*	*Number (cfu g^{-1})*	*Reference*
Listeria monocytogenes	Packaged products sampled at production	France	100	0	<0.04	60
	Packaged products[a]	France	195	0	<0.04	17
	Packaged products sampled at retail	France	605	0	<0.04	399
	Packaged cut green endive	France	41	4.8	NS	77
		France	126	8.8	0.04 to 1	295
	Packaged mixture of lettuce, carrot, and cabbage, sampled at production	Spain	70	30	>0.04	195
	Sliced cucumber, sampled at retail	Malaysia	5	80	NS	29
	Processed vegetables and salads[b]	UK	567	13	NS	353
	Packaged mixed vegetables	UK	60	7	NS	462
		UK	42	19	0.04 to 200	513
	Prepared vegetables and coleslaw sampled at retail[b]	UK	65	4.6	0.04 to 50	328
	Mixed salads of fresh vegetables sampled at retail and in restaurants	US	63	1.6	NS	305
	Ready-to-eat vegetables and salads sampled at retail[b]	Australia	54	1.9	NS	27
	Mixed vegetables[b]	Various European countries	116	3.3 to 44	<100	61
		Germany	20	5	NS	90
	Mixed, fresh vegetables for caterers	Germany	113	0	<0.04	234
	Packaged mixed vegetables sampled at production	Italy	40	0	NS	337
Yersinia enterocolitica	Vegetable salads[b]	Germany	20	0	NS	272
Y. enterocolitica O:3 and O:9	Shredded carrots sampled in restaurants	France	58	7	>0.04	119
Y. enterocolitica	Packaged mixed vegetables sampled at production	Italy	40	0	NS	337
	Packaged products sampled at production	France	100	22 to 56[c]	NS	60
Salmonella	Packaged mixed vegetables sampled at production	Italy	40	0	NS	337
	Packaged products	France	195	0	<0.04	17
	Mixed salads of fresh vegetables sampled at retail and in restaurants	US	63	0	<1	305
	Mixed, fresh vegetables for caterers	Germany	113	0	<0.04	234
Campylobacter	Vegetable salads[b]	Germany	20	0	NS	272

continues

Table 25-9 Continued

Microorganisms	*Product*	*Country*	*Number of Samples*	*Incidence (%)*	*Number (cfu g^{-1})*	*Reference*
Escherichia coli	Mixed, fresh vegetables for caterers	Germany	113	0	<200	234
	Packaged mixed vegetables sampled at retail	UK	12	25	<500	93
	Packaged products sampled at retail	Switzerland	60	0	<10	342
	Packaged products	France	195	30	10 to 100	17
	Mixed salads of fresh vegetables sampled at retail and in restaurants	US	63	12.7	NS	305
E. coli 0157:H7	Mixed salads of fresh vegetables sampled at retail and in restaurants	US	63	0	<0.1	305
Staphylococcus aureus	Packaged mixed vegetables sampled at production	Italy	40	0	NS	337
	Mixed, fresh vegetables for caterers	Germany	113	0	<200	234
	Packaged products sampled at retail	Switzerland	60	0	<100	342
	Packaged mixed vegetables sampled at retail	UK	12	0	<20	93
Aeromonas hydrophila	Packaged mixed vegetables sampled at production	Italy	40	0	NS	337
	Vegetable salads and coleslaw sampled at retail[b]	New Zealand	14	7	0.05 to 100	242
	Vegetable salads sampled at retail[b]	UK	97	21.6	>0.1	192
Bacillus cereus	Mixed, fresh vegetables for caterers	Germany	113	0	<200	234
Clostridium botulinum	Packaged products sampled at production	US	1118	0.36	NS	304

[a] The same survey found 4% of vegetable salads with dressing contaminated with *Listeria monocytogenes*.

[b] Description of the product does not specify the presence of packages, of dressing or of ingredients other than fresh vegetables in the salads.

[c] Strains not pathogenic to man.

NS = Not stated.

grow on most fresh vegetables. However, it grew at 22°C on shredded bell pepper[215] and at 37°C on packaged mushrooms.[97] One hundred *S. aureus* per g produced toxin after 18 hours at 35°C on mushrooms.[340] On minimally processed tomatoes with a pH of 4.1, numbers of *L. monocytogenes* decreased slightly (Figure 25-4C) at 10° and 21°C,[74] whereas *Salmonella montevideo*,[525,542] *Salmonella infantis*, *S. enteridis*, and *S. typhimurium*[30] survived for at least 9 days at 5°C and grew at 20° or 30°C on tomato slices with pH from 3.9 to 4.5. In contrast, *S. montevideo* declined in numbers in the juice of the same tomatoes.[525] The overall measurent of pH presumably did not represent the conditions on tomato slices at the microscopic level.

High storage temperatures permit a rapid growth of bacterial pathogens, but at the same time they accelerate spoilage development and reduce shelf life. Conversely, refrigeration delays spoilage and may increase time for growth of psychrotrophic pathogenic bacteria. Therefore, the important information is the extent of growth or survival of pathogens at time of spoilage development. On shredded green endive, *L. monocytogenes* reached numbers slightly higher at the on-

Table 25-10 Examples of Contamination of Minimally Processed Vegetables of Industrial Origin during Processing

Product	Microorganism	Origin of Contamination	Observation	Reference
Shredded vegetables	Aerobic mesophilic bacteria	Shredders	10- to 100-fold increase of numbers after shredding	198
Shredded lettuce and endive	Aerobic mesophilic bacteria, lactic acid bacteria	Shredders	10- to 100-fold increase of numbers after shredding	259
Sliced carrots	Fecal coliforms	Cutting equipment	10- to 100-fold increase of numbers after cutting	502
Shredded cabbage	*Listeria monocytogenes*	Shredders	Same serotype found on shredders and in the product	295
Various products	*L. monocytogenes*	NS	Incidence higher on processed products than on individual ingredients	90, 513
Shredded iceberg lettuce	*Shigella sonnei*	Food handlers suspected	Outbreaks involving 347 persons traced to shredded lettuce	142

NS = Not stated.

set of spoilage during storage at 10°C and 6°C than at 3°C (Figure 25-5). The bacterium increased in numbers by 100-fold or less before visible spoilage, for storage temperature of 3° to 20°C.[111] However, appreciation of spoilage is subjective. It is difficult to estimate at which spoilage level a product will be rejected by consumers, and therefore to which level of pathogenic bacteria or toxin the consumer might be exposed. For example, respiration of vegetable tissues in packages may create anaerobic conditions and permit toxin production by *C. botulinum* at temperatures of 15°C or above (Table 25-11). Below 15°C, toxin production was usually not observed even with samples inoculated with nonproteolytic *C. botulinum*.[332,396] At temperatures that permitted toxin production, spoilage was observed within a few days, and when toxin was detected, products were considered unfit for consumption in some studies or fit for consumption in others (Table 25-11).

Enteric viruses survived on minimally processed vegetables for several days. From 10^5 pfu g^{-1}, rotavirus inoculated on cut carrots, lettuces, and radishes declined in numbers but were still detectable after 25 to 30 days at 4°C and after 5 to 25 days at room temperature[36]; poliovirus decreased by less than 1 log cycle in 6 to 10 days at 4°C on various cut vegetables and on mixed green salads.[140]

The fate of foodborne pathogenic microorganisms may vary with the vegetable. On minimally processed green salads, *L. monocytogenes* grew faster on butterhead lettuce (*Lactuca sativa*), than on green endive (*Cichorium endivia*), and did not grow on lamb's lettuce (*Valerianella olitoria*).[108] Among a range of shredded vegetables, *S. aureus* grew only on bell pepper.[215] Rotavirus survived for longer periods on lettuce than on radish and carrot.[36]

Other factors may influence growth of a foodborne pathogen on single minimally processed vegetables. The nature of the strains was found important for *C. botulinum*; spores of type A but not of type B strains produced toxin on shredded cabbage.[469] In contrast, the various strains of *L. monocytogenes* tested behaved similarly on minimally processed vegetables.[73,111] *L. monocytogenes* reached higher numbers on minimally processed endive leaves undergoing spoilage, presumably because more nutrients were released.[111] The fate of pathogenic bacteria may also be influenced by amount of inoculum used to measure its growth on minimally processed vegetables. This point is particularly important because most experiments used inoculum levels higher than 10^3 cfu g^{-1}, whereas numbers of pathogens found naturally on vegetables are markedly lower. For example, at 12°C, growth of *E. coli* O157:H7 occurred on various vegetables from an inoculum of 5.0 to 5.3 log cfu g^{-1} but the bacterium declined with an inoculum of 2.2 to 2.5 log cfu g^{-1}.[1] Therefore, results obtained experimentally from

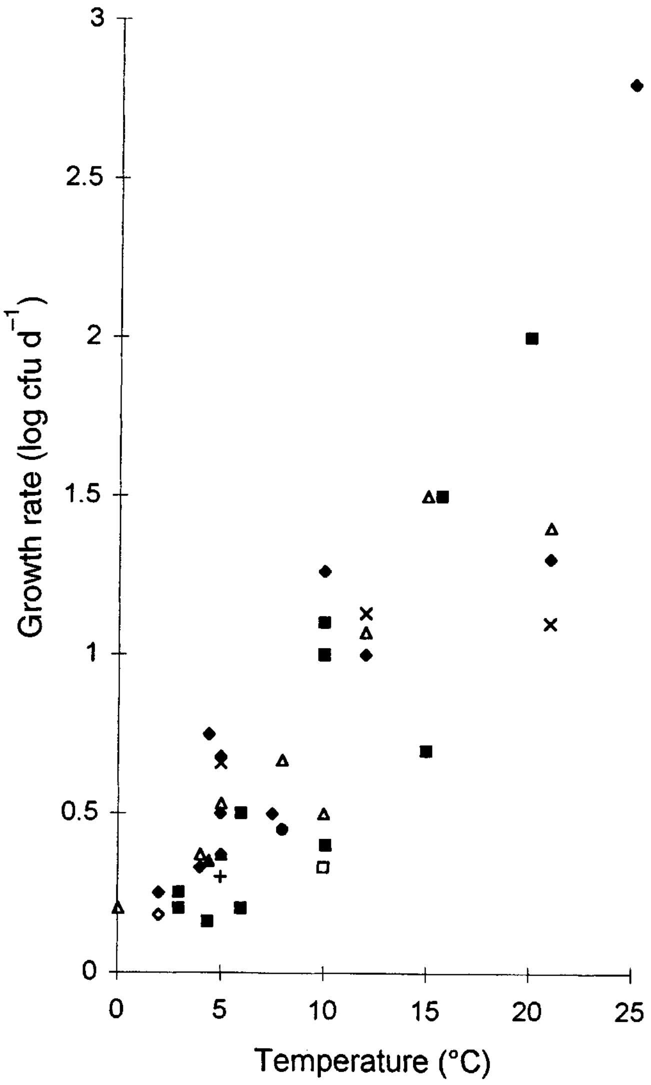

Figure 25-1 Growth Rate of Aerobic Mesophilic Bacteria on Minimally Processed Vegetables As a Function of Storage Temperature. Growth rate was calculated directly, without fitting to a growth model, from the data provided in the references.[1,47,73,74,100,107,111,241,251, 269,280,364,379,405,486,532] Lettuce ◆, endive ■, celery ▲, cucumber ✖, carrots △, broccoli ●, cabbage +, tomato □, onion ◇.

high inocula may not always be extrapolated to the low natural populations. Possible explanations of this are: (1) vegetable surfaces only present a discrete number of sites favorable for growth or survival and low inocula may reduce the probability for the bacteria to colonize these sites, (2) in adverse conditions high numbers of bacteria may create their own favorable environment, or (3) low inocula may fail to compete with other microorganisms.

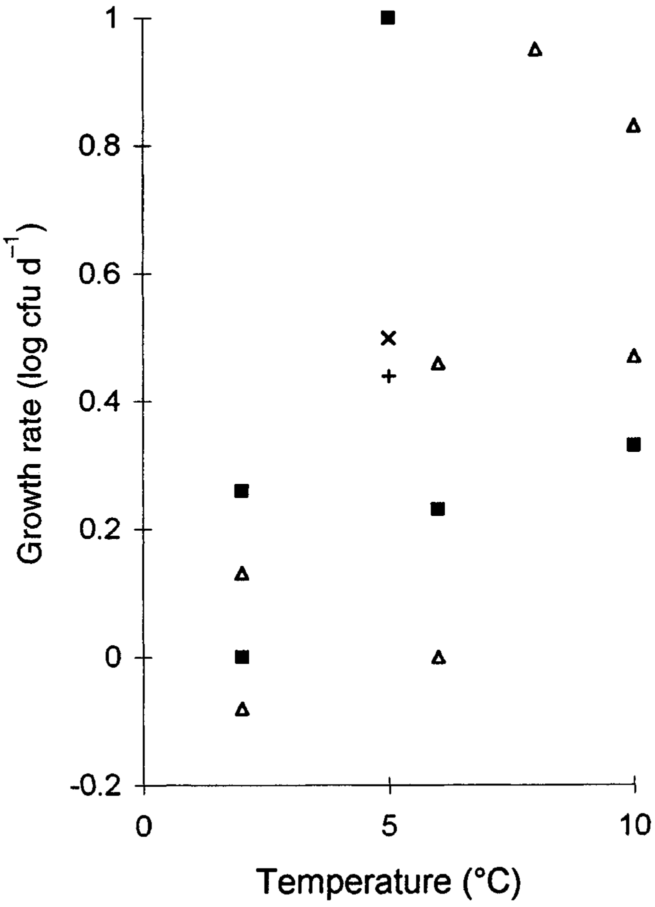

Figure 25-2 Growth Rate of Lactic Acid Bacteria on Minimally Processed Vegetables As a Function of Storage Temperature. Growth rate was calculated directly, without fitting to a growth model, from the data provided in the references.[75,107,117,339,379] Endive ■, carrot △, cabbage +, mixed vegetables ✖.

Effect of Processing

Cutting, slicing, or shredding vegetables probably creates favorable conditions for microbial growth. Aerobic mesophilic bacteria grew faster and reached numbers approximately 10-fold higher on cut carrots, cut celery, and shredded lettuce leaves than on the intact vegetables.[280,405] In contrast, Beuchat and Brackett[73] found the same growth of bacteria on shredded and on whole leaves of iceberg lettuce. Wounds may release antimicrobial factors that are not present on the surface of intact vegetables. This is obvious in the case of tomato because the low pH of internal tissues is detrimental to many bacteria. Wounds may also allow contact between enzymes and substrates and thus release antimicrobials.[136,318]

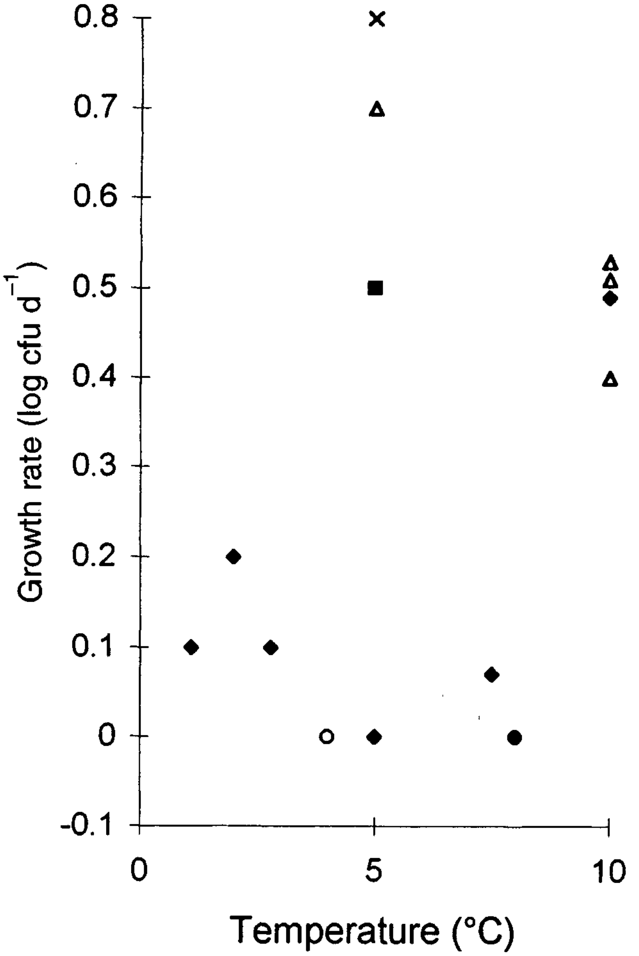

Figure 25-3 Growth Rate of Yeasts and Molds on Minimally Processed Vegetables As a Function of Storage Temperature. Growth rate was calculated directly, without fitting to a growth model, from the data provided in the references.[34,73,280,339,364,454] Lettuce ◆, endive ■, carrot △, broccoli ●, fennel ○, mixed vegetables ✖.

In *Allium* and *Brassica* spp., enzymatic hydrolysis of sulfur amino acid compounds released antimicrobials: allicin and related compounds in the case of onion and garlic[120] and methyl methanethiosulfinate in the case of cabbage.[128,293,294] Hydrolysis products from glucosinolates[84,506] in brassicas also have antimicrobial properties. However, antimicrobials of this kind have not been found to limit growth of microorganisms in minimally processed vegetables. In shredded carrots, an antimicrobial released by cutting the vegetable tissue inhibited *L. monocytogenes.*[72,379,380] Following inoculation onto shredded carrots the bacterium was killed, and in the presence of oxygen, cut or macerated peeled carrot tissue produced a factor lethal to *Listeria* spp. but not lethal to the indigeneous microflora.

Other responses of plant tissues to processing may influence microbial multiplication. Within a few hours after shredding, carrot tissue reabsorbed the exudate released during shredding.[115] Synthesis of membrane lipids occurred after shredding of carrots, indicating a likely repair mechanism.[398] Shredded carrot also accumulated phenolic compounds, which did not show antimicrobial activities but which are precursors of lignin, in a few hours at 4°C,[33] and lignification of peeled carrot surfaces occurred.[82,240] Both reabsorption of exudate and accumulation of phenolic compounds were inhibited under excessive carbon dioxide concentrations or in the absence of oxygen,[33,115] conditions that also permitted growth of lactic acid bacteria on shredded carrot tissues.[115] These results suggest that wound healing processes may limit microbial development on minimally processed vegetables.

25.4.4 Microbial Spoilage of Minimally Processed Vegetables

Spoilage of minimally processed vegetables is characterized by brown discolorations,[40,83,269,280,348,454] necrosis and soft rot,[381] loss of texture, and production of off-odors.[100,116,269] Spoilage appears during storage as the number of microorganisms increases, but attempts to correlate these phenomena have given conflicting results. High microbial loads on unstored shredded lettuce were related to a short shelf life.[83] For stored samples of shredded endive, there was a negative correlation between total bacterial counts and quality.[235] A threshold of 7.4 log cfu g^{-1} separated well-preserved and spoiled samples. However, the relation between spoilage and microbial counts may vary with storage temperature (Table 25-12), either because different microorganisms became dominant at different temperatures or because other factors participate in spoilage. It may also vary with the kind of vegetables. Batches of shredded broad-leaved endive with a large amount of spoilage contained a higher proportion of pectinolytic fluorescent pseudomonads.[381] In contrast, pectinolytic pseudomonads were present in equal numbers in spoiled and unspoiled samples of shredded carrots and spoilage at 10°C was associated with high numbers of lactic acid bacteria.[116]

Inoculation of 10^7 cfu g^{-1} of some strains of pectinolytic fluorescent pseudomonads caused soft rot on shredded endive within 4 days at 10°C[254,381] and shredded carrots inoculated with *Leuconostoc*

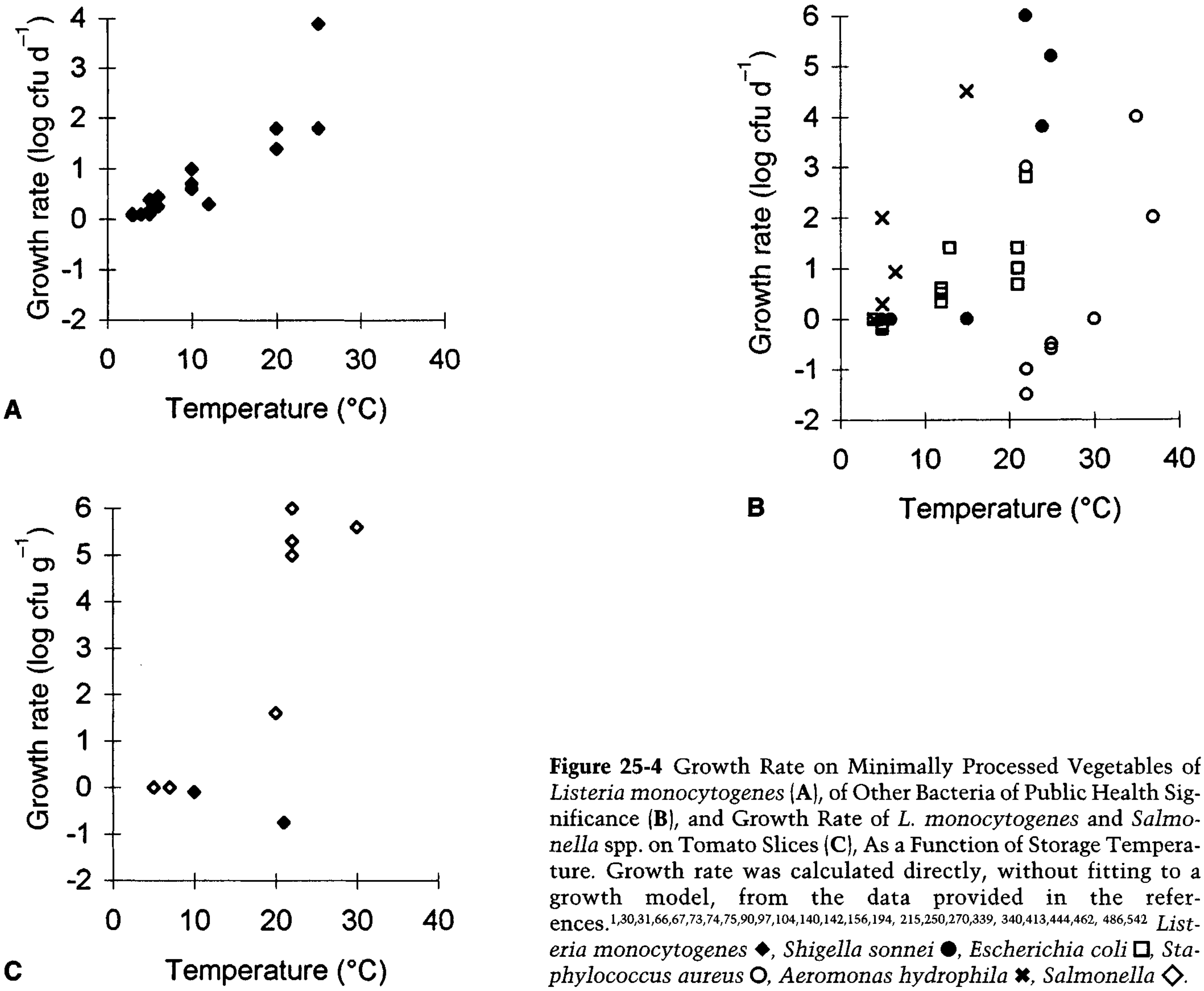

Figure 25-4 Growth Rate on Minimally Processed Vegetables of *Listeria monocytogenes* (**A**), of Other Bacteria of Public Health Significance (**B**), and Growth Rate of *L. monocytogenes* and *Salmonella* spp. on Tomato Slices (**C**), As a Function of Storage Temperature. Growth rate was calculated directly, without fitting to a growth model, from the data provided in the references.[1,30,31,66,67,73,74,75,90,97,104,140,142,156,194, 215,250,270,339, 340,413,444,462, 486,542] *Listeria monocytogenes* ◆, *Shigella sonnei* ●, *Escherichia coli* □, *Staphylococcus aureus* O, *Aeromonas hydrophila* ✖, *Salmonella* ◇.

mesenteroides reproduced the same symptoms as those observed on naturally spoiled samples (ie, exudation and off-odors).[115] However, spoilage on endive leaves inoculated with pectinolytic pseudomonads was reduced under 20% carbon dioxide,[113] and, conversely, *L. mesenteroides* produced symptoms on shredded carrots only under carbon dioxide concentrations above 20% or oxygen concentrations below 2%.[115] Microorganisms may be a cause of spoilage but storage conditions are a determining factor.

Evidence of the role of microorganisms in spoilage of minimally processed vegetables is scant. The reasons may be: (1) cut vegetable tissue can probably withstand multiplication of many microbial species without damage, presumably because of healing processes, (2) minimal processing causes many changes in the vegetable tissue, which may lead to spoilage more rapidly than microorganisms, these changes include increased respiration rate, delocalization of cell compartments, oxidative degradation of substrates, and toxicity of overmodified atmospheres.[509]

Microorganisms causing spoilage of raw, unprocessed vegetables have been isolated from minimally processed vegetables; these microorganisms include *Sclerotinia* spp., *Mucor* spp., and *Pseudomonas viridiflava* in shredded carrots,[116] and pectolytic *Erwinia* in mixed vegetable salads.[93] These microorganisms have never been implicated in spoilage of

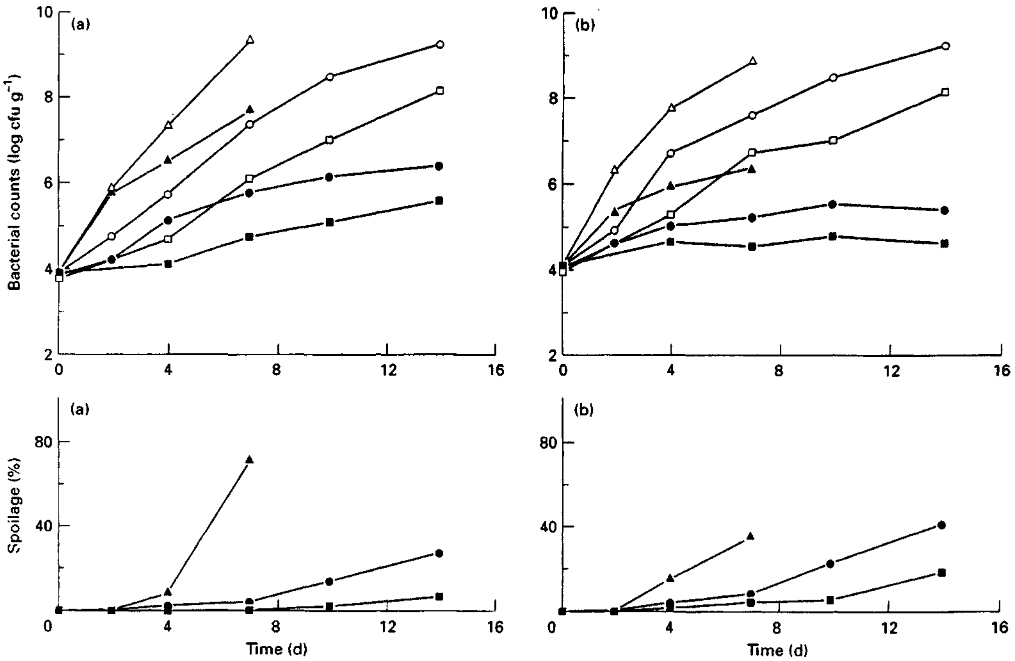

Figure 25-5 The Effects of Storage at 3°C (■, □), 6°C (●, ○), and 10°C (▲, △) on the Population of *Listeria monocytogenes* (closed symbols) and Aerobic Bacteria (open symbols), and on the Extent of Spoilage on Leaves of Shredded Endive at Two Different Times of the Year (a and b). Shredded endive was packaged in a polypropylene film and spoilage was measured by the surface of decay in % of the leaf surface. *Source:* Reprinted with permission from F. Carlin, C. Nguyen-the, and A. Abreu da Silva, Factors Affecting the Growth of *Listeria Monocytogenes* on Minimally Processed Fresh Endive, *Journal of Applied Bacteriology*, Vol. 78, p. 640, © 1995, Society for Applied Microbiology.

minimally processed vegetables, probably because shelf life is too short, compared with the storage of raw vegetables.

25.5 CONTROL OF MICROORGANISMS ON FRESH VEGETABLES

25.5.1 Prevention of Contamination

Polluted water, sewage effluent, manure, or other sources of organic fertilizers are known vectors of microorganisms pathogenic to man or animal, and control of agricultural practices are necessary to prevent contamination. Between 1935 and 1980 in Jerusalem, the incidence of cholera and helminth infections dropped dramatically when supply of vegetables irrigated with polluted water was cut off, and when this practice was eventually stopped.[460] In Peru, cabbage irrigated with polluted water was also a source of cholera.[492] Measures taken to prevent cholera in Chile and in Costa Rica during the 1991–1993 epidemics included destruction of vegetables irrigated with sewage-contaminated water and putting emphasis on wastewater treatments.[343] Health authorities have usually been aware of this risk, and in many places, irrigation of vegetable crops with untreated wastewaters is banned, although enforcement of such measures may be problematic. Wastewater treatments usually reduce numbers of foodborne pathogens.[261] However, in the US, the incidence of rotavirus was similar in raw and treated sewage, whereas the incidence of enterovirus was efficiently reduced.[463] In The Netherlands, *L. monocytogenes* was present in 67% of sewage treatment plant effluents,[158] and in Italy the frequency of *L. monocytogenes* was similar in treated and untreated wastewater, although treatment reduced the number of listeria 100-fold.[65] Microbial pathogens eliminated from sewage would be concentrated in the sludge. For example, *L. monocytogenes*[209,449] and thermotolerant *Campylobacter*[390] are frequent in sewage sludge. However, digestion of sludge may reduce the number of pathogenic microorganisms.[450] Pollu-

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Table 25-11 Examples of Toxin Production by *Clostridium botulinum* in Packaged, Fresh Vegetables

Product	*Storage Temperature (°C)*	*Initial Head Space in the Package*	*Inoculum (spores)*	*Time to Toxin Detection (days)*	*Product Quality at Time to Toxin*	*Reference*
Romaine lettuce	21	Air	100 g^{-1}	14 to 21	Inedible	396
Shredded cabbage	21	Air	100 g^{-1}	7	Inedible	396
	22 to 25	70% CO_2, 30% N_2	100 to 200 g^{-1}	4 to 6	Acceptable	469
Sliced fresh potatoes	22	Vacuum	550 g^{-1} (type A)	3	Marginally acceptable	470
Sliced potatoes with sulfite (136–174 ppm)			3000–3750 g^{-1} (type A)	4	Acceptable	
Agaricus bisporus mushroom	20	Air	10^4 per mushroom	3 to 4	Edible	491
Flammulina velutipes mushrooms	15	Vacuum	1 g^{-1}	14	Spoiled	332
	21	Vacuum	0.1 g^{-1}	21	Spoiled	
	27	Vacuum	10 g^{-1}	2	Slight spoilage	

tion of surface waters may also be a cause of contamination of vegetable crops. A limit of 1,000 fecal coliform 100 ml^{-1} for irrigation water for vegetables was proposed in the European Union and in the US.[207,261] Disinfection of irrigation waters was also proposed,[261] for instance, by UV treatments,[426] although turbidity of water and fouling may be a problem. Drip irrigation associated with plastic lining of soil also significantly reduced contamination of aerial parts of crops by polluted water[438] compared with spray irrigation. The capacity of some pathogens to survive for several days in cultivated soil, at least 8 weeks for *L. monocytogenes* in soil contaminated with sewage sludge,[523] must be taken into account.[98,390]

Table 25-12 Numbers of Aerobic Mesophilic Bacteria and Spoilage on Minimally Processed Endive Packaged in Sealed Polypropylene Film

Storage Temperature (°C)	*Highest Bacterial Counts before Noticeable Spoilage (log cfu g^{-1})*	*Bacterial Counts When Spoilage Was First Noticeable (log cfu g^{-1})[a]*
3	7.0	8.1
	7.0	8.1
6	7.2	8.2
	7.7	8.3
10	6.0	7.2
	6.2	7.8
20	6.1	7.2
	5.5	6.2

[a] Spoilage became noticeable when approximately 2% of the leaf surface showed decay.

Source: Reprinted with permission from F. Carlin, C. Nguyen-the, and A. Abreu da Silva, Factors Affecting the Growth of *Listeria Monocytogenes* on Minimally Processed Fresh Endive, *Journal of Applied Bacteriology*, Vol. 78, pp. 636–646, © 1995, Society for Applied Microbiology.

Microbial spoilage after harvest frequently results from disease initiated in the field (Section 25.3.2). In such cases, reduction of infection in the field may reduce post-harvest decay. The use of disease-free seeds may reduce post-harvest spoilage. Seed certification schemes exist for *Clavibacter michiganense* on potato tubers.[466] Precautions are necessary to prevent recontamination of the crop by gangrene, silver scurf, skin spot, and blackleg during multiplication of seed potatoes.[310] Dead parts of the plant may be a primary source of inoculum and removal of necrotic tissues on onion leaves in the field reduced significantly both the level of airborne conidia of *Botrytis* spp. and the incidence of the disease.[238] It is frequently advisable to destroy crop debris after harvest to eliminate sources of inoculum for the next crops, for example, to eliminate fungal pathogens of potatoes or carrots.[466] Crop rotation may also help to prevent build-up of inoculum, but this may be hampered by the broad host range of some diseases, which may include weeds, as for *Sclerotinia sclerotiorum*,[81] and by the long survival of some forms of inoculum such as sclerotia of *Rhizoctonia* and *Sclerotinia* spp.[466] or of *Sclerotium rolfsii*.[409] Chemical treatment, solar heat, or microbial antagonist were proposed to reduce sclerotia in the soil.[208,409,466] Reduction of numbers of *Erwinia carotovora* on roots of potato plants, by introducing microbial competitors in the rhizosphzere, reduced incidence of soft rot on stored tubers.[536] Fungicide treatments to protect foliage against *Sclerotinia sclerotiorum* before harvest reduced infection on carrot roots during storage.[406] Such strategies have not always been successful,[434] and the use of fungicides near harvest is strictly regulated. Conditions favoring infection of the plant may be reduced by agricultural practices.[395,409,466] These practices include deep ploughing to bury sclerotia below the layer of soil used by carrot roots; use of cultivars with upright growth to minimize contact of foliage with soil as in the cases of green salads or carrots[406]; protecting foliage from soil by plastic lining and avoiding splashes during irrigation; preventing formation of a humid microclimate by overgrowth of canopy; proper draining of soil to prevent waterlogging;[395] and minimizing mechanical damages at harvest in the case of wound spoilage agents.[168,320]

Prevention of contamination in packing houses, storage facilities, or processing lines relies on a proper cleaning and disinfection of premises and equipment. This is the main objective of the Code of Good Manufacturing Practices for minimally processed fresh vegetables,[24] together with a physical separation of the various steps and a forward progression to avoid cross-contamination of the end-products with wastes or with dirt brought in by raw vegetables.[15] The application of the European Directive 93/43/CEE in European Union member states will lead to regulation of the hygiene of all food products, including storage of raw vegetables. Post-harvest diseases were reduced by disinfection of equipment in contact with vegetables,[51,257] but the main point is disinfection of water used to wash, to cool, or to flume vegetables to avoid build-up of contamination. In carrot packinghouses, 2 to 25 mg l^{-1} chlorine prevented a build-up of *E. carotovora* in the wash water and reduced the incidence of soft rot during storage[452]; similar results were obtained on tomato fruits.[49] Chlorine may be supplied as the gas or as hypochlorite. Its activity is increased at low pH, whereas it is more stable at high pH. A pH between 6 and 7 is an optimum between stability and activity.[319] Fungal propagules need a higher concentration than bacteria to be inactivated, the minimum concentration of free available chlorine reducing the numbers of viable cells by 6 log cycles in 2 minutes at 25°C at pH 6 in water was 0.4 mg l^{-1} for *E. carotovora* and 25 mg l^{-1} for *Geotrichum candidum*.[424] The level of free available chlorine must be monitored and adjusted repeatedly because it combines rapidly with organic material.[51,172,173,315,319] The level of active chlorine is one of the critical points identified in codes of good manufacturing practices for minimally processed vegetables.[24] Alternatively, ozone[482] or chlorine dioxide was proposed[139,416]: concentrations of 0.3 ppm of ozone had a similar effect to 54 ppm chlorine on fungal propagules, and 1.3 ppm of chlorine dioxide was found sufficient to control bacteria in hydrocooling water, whereas higher concentrations produced an excessive odor.

25.5.2 Reduction of Contamination

Destruction of microorganisms that contaminate vegetables relies mostly on chemical treatments, although physical treatments have also been tested. The purposes may be to reduce the general contamination, to remove foodborne pathogens, or to reduce microbial spoilage. Compared with the *in vitro* effect of antimicrobial agents, their use on vegetables is limited by several factors: (1) the microorganisms may not be accessible to the antimicrobial agent, (2) the agent may be phytotoxic, (3) whenever applied as an aqueous solution, free water may remain on the vegetable surface, which may permit spoilage development, and (4) the agent may not be authorized for toxicological or environmental reasons or may not be accepted by consumers.

Water rinsing alone reduces bacterial contamination of vegetables by less than 1 log cycle.[377] It usually has no effect on microbial spoilage,[51] and may even increase contamination or facilitate penetration of microorganisms as discussed above for soft rot *Erwinia* spp. on potatoes or tomatoes. However, washing carrots in water at harvest reduced the incidence of microbial spoilage during storage by a factor of 3 to 5.[309] Among sanitizers, chlorine is widely used, but in spite of its efficiency on microorganisms suspended in water, its effect on the number of microorganisms on vegetables is limited (Table 25-13).[89,377] Sixty percent of the initial level of free available chlorine (100 mg l^{-1}) was found after treatment of lettuce leaves, and microorganisms surviving disinfection were not resistant to the residual level of free available chlorine.[4] The surface-active agent Tween 80 (80 to 100 mg l^{-1}) improved the activity of 100 mg l^{-1} chlorine slightly but caused deterioration of lettuce leaves.[4] Increasing the chlorine concentration above 100 mg l^{-1} and prolonging contact time above 30 minutes did not improve disinfection (Table 25-13). Chlorine concentrations above 300 mg l^{-1} may cause oxidation of equipment and may be an irritant to employees.[4]

Upon reaction with organic matter, chlorine may produce potentially hazardous trihalomethanes. For this reason, and because of a more general concern about the impact of chlorine on the environment, its use for treatment of minimally processed vegetables is banned in several European countries, including Germany, The Netherlands, Switzerland, and Belgium (Assoc. Fr. Fabr. 4ème gamme, pers. comm.). Chlorine dioxide, hydrogen peroxide (Table 25-13), and ozonated water[387] reduced numbers of microorganisms on vegetables but were not more efficient than chlorine.

Microbial populations surviving disinfection grew faster than the corresponding populations on the nondisinfected products, eventually reaching the same numbers, and reduction of numbers of microorganisms by disinfection did not increase shelf life (Table 25-13).[73,112,377,391] The normal saprophytic microflora of endive leaves was antagonistic to *L. monocytogenes*,[112] and *L. monocytogenes* grew faster on endive leaves with an epiphytic population reduced by disinfection.[112] However, disinfection reduced the number of *L. monocytogenes* present on the raw material (Table 25-13). In addition, reduction of numbers of nonpsychrotrophic pathogens by disinfection would persist until consumption if refrigeration during storage is properly maintained. Microbial spoilage of unprocessed vegetables was reduced by some disinfectants (Table 25-14) but concentrations needed were higher than those used to reduce the number of microorganisms (Table 25-13).

Rather than reducing the number of microorganisms, a disinfectant may be more useful in preventing build-up of microorganisms on equipment and in water and to avoid dissemination from contaminated materials. Lower concentrations of disinfectant would probably be sufficient. However, in industrial conditions the large volume of water used to wash vegetables makes on-line control of concentration difficult; it may be advisable to keep the target value higher than the minimum necessary.

Irradiation with doses ranging from 0.5 to 2 kGy had no adverse effect on products stored a few days under refrigeration as minimally processed vegetables.[89,377] At 2 kGy, numbers of bacteria were usually reduced by 3 to 4 log cycles and yeasts by 1 to 2 log cycles. Irradiation seems therefore more efficient than sanitizers. In contrast, most attempts to reduce post-harvest microbial spoilage of raw vegetables failed because the dose required to inactivate spoilage agents, and more particularly fungal propagules, had adverse effects on the product.[346] The shelf life of raw vegetables is longer than that of minimally processed products, which may allow the development of damage induced by irradiation.

Attempts have been made to inactivate microorganisms by mild heat treatment.[43] In aqueous suspension, only 18.3% of spores of *Rhizopus stolonifer* germinated after exposure to 49°C for 2 minutes[465] and 72 hours at 38°C caused a 100% inhibition of *B. cinerea*. This latter treatment inhibited decay on tomatoes treated at the pink stage of maturity, without causing significant modification of the product.[182] However, heat treatments needed to inactivate spoilage agents are very close to the limit of injury to vegetable tissues,[173] and could facilitate attack by microorganisms.

25.5.3 Inhibition of Microorganisms by Chemical and Biological Agents

Post-harvest application of fungicides efficiently reduces spoilage,[153,172] but many spoilage fungi such as *Rhizopus* spp., *Mucor* spp., and *Geotrichum* spp. are resistant to a large number of fungicides.[171,172,365] On carrots post-harvest, benomyl and iprodione dips reduced incidence of *B. cinerea* and *S. sclerotiorum* but failed to control *R. carotae*.[154,204] Resistant strains appear among species normally sensitive,[436] for example, *B. cinerea*[64,308,383,481,520] and potato tuber fungal pathogens.[118] Only a few post-harvest applications of chemicals have been approved by legal authorities, with substantial differences between countries.[2,184]

Natural chemicals have been tested as alternatives to synthetic fungicides. The essential oil carvone in the storage atmosphere reduced decay of potato tubers inoculated with *Phoma exigua* but not with *Fusarium solani*[229]; on bell pepper injured and inoculated with *B. cinerea*, a coating with chitosan reduced decay.[176] Microbial antagonists are another possible alternative (Table 25-15).[167] In the studies on onion, tomato, and cabbage (Table 25-15), antagonists were isolated from the vegetables themselves, indicating that the epiphytic microflora of vegetables could be a natural barrier against microbial spoilage. *Pseudomonas cepacia* mostly acts through the production of the antifungal coumpound pyrrolnitrin.[101] In this case, problems are likely to arise from the appearance of resistant strains among the spoilage microorganisms. The mode of action of other antagonists has not been fully explained. It probably involves competition for nutrients, direct interaction with the pathogen (ie, adhesion to the

Table 25-13 Effect of Disinfectants on the Microbial Contamination of Vegetables

Vegetable	*Disinfectant*	*Concentration (mg l^{-1})*	*Conditions*	*Reduction of Microbial Load upon Disinfection (log cfu g^{-1})*	*Residual Reduction after Storage*	*Reference*
Peeled carrots[g]	Sodium hypochlorite	20	20 min, 10°C	0.5 for aerobic mesophilic bacteria[a]	NS	502
				>1.3 for coliform and fecal coliform[a]	NS	
Shredded iceberg lettuce[f]	Sodium hypochlorite	200 to 250	10 sec	1 to 2 for aerobic mesophilic bacteria[a]	No reduction after 4 d at 10° or 5°C	73
Shredded cabbage[g]	Chlorine	300	Ice water	No effect on aerobic mesophilic bacteria[a]	NS	198
Shredded lettuce[g]				3 for aerobic mesophilic bacteria[a]	NS	198
Cut carrots[g]				No effect on aerobic mesophilic bacteria[a]	NS	198
Lettuce[f]	Sodium hypochlorite	0.2 (tap water)	5 min, pH 7.3[c]	1.1 for aerobic mesophilic bacteria[a]	Reduction diminished on storage	4
		100	5 min, pH 8.8[d]	1.4 to 1.7 for aerobic mesophilic bacteria[a]	After 24 h at 20°C, microbial counts higher than on lettuce washed in water	
		300	5 min, pH 9.4	2.1 for aerobic mesophilic bacteria[a]	NS	
Shredded green endive[h]	Sodium hypochlorite	50 to 200	5 to 20 min, pH 8 to 6[e]	1 to 2 for aerobic mesophilic bacteria[a]	NS	347
Tomato[f]	Sodium hypochlorite	60	2 min, 25°C, pH 7.1	0.6 for *Salmonella*[b]	NS	541
		110	Idem	1.3 for *Salmonella*[b]	NS	
Brussels sprouts[f]	Sodium hypochlorite	0	0.5 min	1.4 for *Listeria monocytogenes*[a]	NS	85
		200	Idem	2.0 to 2.5 for *L. monocytogenes*[a]	NS	
Watercress[f]	Sodium hypochlorite	50 to 500	1 min, 25°C	0.6 to 0.8 for aerobic mesophilic bacteria[b]	No reduction after 6 d at 5°C	391
		1000		1.6 for aerobic mesophilic bacteria[b]	Idem	

continues

Table 25-13 Continued

Vegetable	*Disinfectant*	*Concentration (mg l⁻¹)*	*Conditions*	*Reduction of Microbial Load upon Disinfection (log cfu g⁻¹)*	*Residual Reduction after Storage*	*Reference*
Shredded lettuce[f]	Sodium hypochlorite	200	10 min, 4°C, pH 8.6	1.3 for *L. monocytogenes*[b]	NS	541
	Chlorine dioxide	5	10 min, 4°C, pH 7.4	1.1 for *L. monocytogenes*[b]	NS	
Shredded endive[f]	Hydrogen peroxide	10% (vol/vol)	2 min, 22°C	1.6 for *L. monocytogenes*[b]	No reduction after 4 d at 10°C	112

Note: All disinfectants were applied as dips. Concentrations for chlorine derivatives are expressed as available free chlorine.

[a] Compared with the untreated product.

[b] Compared with water wash.

[c] 20 min gave reduction similar to 100 mg l⁻¹ available free chlorine.

[d] 30 min gave no further reduction. Reduction slightly increased (0.1 to 0.2 log) by lowering pH between 4 and 7.

[e] High variations between experiments. Increasing chlorine concentration from 50 to 200 mg l⁻¹, prolonging contact time, and reducing pH did not improve disinfection.

[f] Results obtained in laboratory.

[g] Results obtained in industrial conditions.

[h] Pilot scale equipment.

NS = Not stated.

Table 25-14 Example of Use of Disinfectants to Reduce Microbial Spoilage of Stored Vegetables

						% Spoiled Products		
Vegetable	*Disinfectant*	*Concentration*	*Conditions*	*Spoilage Agent*	*Storage Conditions*	*Treated*	*Control*	*Reference*
Eggplant	Sanosil-25[a]	0.5% (vol:vol)	1 min, 20°C	*Botrytis cinerea* and *Alternaria alternata*[b]	14 d at 12°C	0	11	181
Red bell pepper						2.5	8	181
Potato tubers	Sodium hypochlorite	1000 mg l^{-1}	5 min	*Erwinia carotovora* pv *carotovora*[c]	4 d at 20°C	22	80	54
Potato tubers	Sodium hypochlorite	1000 to 2000 mg l^{-1}	5 min	*Erwinia carotovora* pv *atroseptica*[c]	5 d at 20°C under anaerobic conditions	30 to 100	80 to 100	324
Potato tubers	Chlorine dioxide	1.5 g l^{-1}	5 min	*Erwinia carotovora* pv *atroseptica*[c]	5 d at 20°C under anaerobic conditions	10	95	535
	Acetaldehyde vapor	30 ml l^{-1} in humidified air	4 h		5 d at 20°C under anaerobic conditions	20	95	

[a] Hydrogen peroxide (48%) stabilized with silver ions.

[b] Natural contamination.

[c] Artificial inoculation.

mycelium of the fungal pathogen and destruction), or induction of defense reaction from the host tissues.[166] The efficacy of microbial antagonists could be improved by the addition of nutrients, salts, and the combined application with fungicides. Approval by legal authorities is one major limitation to the use of such antagonists. However, some microbial antagonists have recently been registered for commercial use in the US.[16,166]

For minimally processed fresh vegetables, inoculation of *Lactobacillus casei* reduced multiplication of mesophilic bacteria.[517] The introduction of *L. casei* caused a significant decrease in pH of the product, and unfortunately, the authors gave no indication of the appearance of treated products.

25.5.4 Inhibition of Microorganisms by Control of Storage Conditions

Refrigeration

Refrigeration is widely used for storage of raw vegetables and of minimally processed fresh vegetables.[368] The need for refrigeration during processing of minimally processed vegetables is specified in codes of good manufacturing practice,[24] and refrigeration during transport and retail sale is a legal obligation in France.[15] The rapid multiplication of many foodborne pathogens on minimally processed vegetables at temperatures above 10°C results in the need for refrigeration. Refrigeration of minimally processed endive reduced the growth rate of *L. monocytogenes* more than that of the aerobic microflora,[111] and therefore the assumption that low temperatures may extend the time available for growth of *L. monocytogenes* before microbial spoilage is presumably not true in the case of minimally processed vegetables. Spoilage by some microorganisms may be prevented by storage at low temperatures[153]: eg, soft rot *Erwinia*,[314] *Sclerotium rolfsii*, *Phytophthora* spp., and *Fusarium* spp.[466]; whereas *B. cinerea*,[3,418] *M. acerina*,[300] and *P. fluorescens*[315] cause spoilage of products stored at temperatures close to 0°C.

The efficiency of refrigeration is limited by several factors:

1. Whenever crops of raw vegetables are transferred directly to a cold room, time to reach the target temperatures in the center of the bulk of product may extend to several days, allowing time for nonpsychrotrophs to develop. Two months was necessary to lower the temperature of carrots from 20° to 2°C at the center of 400 kg bins,[503] and 75 days was necessary to lower the temperature to 1°C in storage rooms of carrot.[406] A rapid cooling is frequently advisable before storage; this can be achieved by hydrocooling, forced air cooling, or vacuum cooling.[362] Reducing the time to cool carrots to 1°C from 72 hours to 6 hours reduced the incidence of *S. sclerotiorum* during storage by a factor of 4.[406] For hydrocooling, sanitation of the cooling water must be properly conducted as discussed above.
2. For products that required refrigeration throughout shelf life, such as minimally processed fresh vegetables, temperature fluctuations are liable to occur during transport and retail or in catering facilities.[345,445]
3. Temperatures low enough to restrict growth of spoilage microorganisms cause injury of some crops. Cucumber, tomato, bell pepper, aubergine,

Table 25-15 Examples of Reduction of Post-Harvest Diseases of Vegetables by Microbial Antagonists

Vegetable	*Spoilage Agent*	*Antagonist*	*Effect on Stored Product*	*Reference*
Carrot	*Rhizoctonia carotae, Mycocentrospora acerina*	*Trichoderma harzianum*	Increase of marketable crop by 47% to 75%	503
Onion	*Botrytis allii*	*T. harzianum*	Complete inhibition of spoilage	427
Tomato	*Botrytis cinerea*	*T. harzianum*	Soft rot incidence reduced by 57%	459
Cabbage	*B. cinerea*	*Pseudomonas fluorescens* and *Serratia liquefaciens*	Losses reduced by 50% to 55% (in weight)	301
Potato	*Fusarium sambucinum*	*Pseudomonas cepacia*	Dry rot development reduced by 50% to 90%	101

yam, sweet potato, and many vegetables of tropical origin may develop injuries such as pitting of the epidermis, scald, and necrosis at temperatures below 10°C or even below 15°C. This leads to an increase in microbial spoilage, either by psychrotrophs during cold storage or when the product is brought back to ambient temperature.[134,152,466] Intermittent warming at 20°C during storage of tomatoes at 9°C reduced cold injury, reduced spoilage (caused by *Phytophthora* spp., *Geotrichum* spp., and *Alternaria* spp.) and delayed ageing compared with continuous storage at 9°C, which caused cold injury, or at 12°C, which did not cause cold injury but did not sufficiently delay spoilage and ageing.[28] This method failed to prevent development of injury at 6°C.

4. Traditional cold rooms may cause dehydration of stored vegetables, which may reduce resistance to microbial infection, and therefore increase spoilage. Carrots incubated at 25°C after cold storage developed infection by *R. stolonifer* only if the roots had lost more than 8% of fresh weight during the storage period.[497] Cooling systems maintaining a high humidity are therefore advisable and minimizing weight loss of carrots by high humidity also reduced fungal decay caused by *Sclerotinia* spp.[505] and *B. cinerea*.[204] However, *M. acerina* can cause devastating losses at high humidity[300] and high humidity favored soft rot of carrots caused by *R. carotae*.[154]
5. Temperature fluctuations may cause condensation, which can facilitate penetration by spoilage microorganisms.

Modification of the Storage Atmosphere

The primary effect of the modified atmosphere is usually to reduce the respiration of the produce and therefore to delay physiological changes, which may also reduce microbial spoilage. The modified atmosphere is either actively generated and controlled in the storage room used for raw vegetables (*controlled atmosphere*), or passively generated by the respiration of the vegetables in a sealed package (*modified atmosphere*). In the latter, the composition of the atmosphere tends toward an equlibrium that is a function of the respiration rate, the permeability of the packaging film, and the initial head space of the package.[268] Increasing the storage temperature or stressing the vegetable tissue, as in minimal processing, increases respiration and the extent of atmosphere modification. For a given product, the desired atmosphere composition at equilibrium may be achieved by selection of the packaging film and a given temperature, and by modifying the initial head space of the package.[509] Modified atmosphere packaging was first applied to whole vegetables and later to minimally processed fresh vegetables.[377,509] Atmosphere modifications are limited by the phytotoxicity of carbon dioxide (oxygen) levels above (below) limits that depend on the vegetable and the storage temperature.[249,268,311,509] For long-term storage of raw vegetables, recommended concentrations of carbon dioxide rarely exceed 5% or 10%, and the oxygen level is usually maintained at 3% to 4%, although new techniques permit the use of ultra-low oxygen concentrations, ie, below 1%. In minimally processed vegetables, accumulation of carbon dioxide above 15% or 20%, and a depletion of oxygen below 1% after a few days at temperatures between 5° and 10°C were frequently reported, but this concerns products with short shelf life.[509]

Either oxygen reduction below 5% or increase in carbon dioxide above 10% delays growth *in vitro* of aerobic microorganisms present on vegetables (Table 25-16).[177] No complete inhibition was recorded and atmosphere modification had no remaining effect after transfer of microorganisms to air. Controlled atmosphere reduced microbial spoilage of vegetables like tomato, cabbage, and celery (Table 25-17), although some spoilage agents were not inhibited. Reduction of spoilage may involve mechanisms other than inhibition of growth of spoilage microorganisms. For instance, carbon dioxide inhibited maceration of endive leaves by a culture filtrate of pectolytic *P. fluorescens*.[113,509]

On the contrary, microbial spoilage of potato and carrot was increased, although microorganisms tested were sensitive *in vitro* to atmosphere modifications. Defense mechanisms of the vegetable tissue were probably reduced, as suggested by the greater ability of *E. carotovora* to grow in potato tubers, and by the higher susceptibility of potato tubers to maceration by pectic enzymes of the bacterium, under anaerobiosis.[331] Similarly, atmospheres with increased carbon dioxide or reduced oxygen levels inhibited characteristic reactions of the wound response of carrot tissues, such as accumulation of phenolic compounds and increase in phenyl ammonia lyase activity.[33]

Storage under modified atmosphere packaging reduced microbial spoilage of some raw vegetables (Table 25-18), but, in some cases, the modifications in gas composition were so slight that the positive effect may have been the result of prevention of water loss by the package. Conversely, the high humid-

Table 25-16 Effect *in vitro* of Modified Atmospheres on Growth of Microorganisms That Are Commonly Found on Vegetables. Atmosphere Modifications Considered Are Those Compatible with Storage of Fresh Vegetables

Microorganisms	*O_2 : CO_2 (%)*	*Temperature (°C)*	*Effect Compared with Air*	*Reference*
Fungi				
Aspergillus niger, Mucor hiemalis, Penicillium expansum, Rhizopus oryzae	2 : 10.5	16 and 21 1 and 7	No effect Two-fold increase in lag time, no effect on growth rate	537
Cladosporium herbarum, Alternaria spp.	2 : 10.5	16 and 21 7	Slight effect Increase in lag time and 10-fold reduction in growth	537
Sclerotium rolfsii	21 to 19 : 1 to 3 13 to 10 : 9 to 12	30°C	Stimulation of growth Five-fold reduction in growth rate	291
Fusarium moniliforme, Rhizopus spp., *Aspergillus ustus*	0.1 : 0 1 : 0 0.1 : 0 to 4	25	Reduction of growth between 84% and 90% Reduction of growth between 20% and 50% CO_2 stimulated growth at low O_2	211
Botrytis cinerea	1.5 : 4 1.5 : 0	1	Delay in spore germination and in elongation of spore germ tubes, but no effect on mycelium No or only slight effect	419
Alternaria tenuis, Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum	21 to 0 : 4.7	24	Significant reduction in growth for 0.8% or less O_2	3
Fusarium roseum			No effect	
Geotrichum candidum	1 or 3 : 0 or 3 0 to 1 : 0 or 3	21 21	Stimulation of growth Reduction of growth	528
Bacteria				
Pseudomonas fluorescens	17 to 7 : 5 to 15	11.5	Increase in lag time and generation time with increasing CO_2; five-fold increase in lag time and 1.5-fold increase in generation time at 15% CO_2	532
P. fluorescens	21 : 0 to 10 21 : 10 to 30 21 to 0 : 0	21	Slight reduction in growth Reduction of growth by 85% at 30% CO_2 Reduction of growth by 50% and by 90% in 3% and 0.5% O_2, respectively	527
Pseudomonas marginalis	3 : 10	20	No effect on growth rate, lag time increased from 17 h in air to 24 h	46

continues

Table 25-16 Continued

Microorganisms	*O_2 : CO_2 (%)*	*Temperature (°C)*	*Effect Compared with Air*	*Reference*
P. fluorescens, *P. marginalis*	1 to 4 : 0 to 10	12.5	Lag time increased; no effect on growth rate	244
Erwinia carotovora, *E. atroseptica*	21 : 0 to 3 21 : 3 to 30 21 to 0 : 3	21	Stimulation of growth Reduction of growth by 85% at 30% CO_2 Reduction of growth by 40% and by more than 80% at 3% and 0% O_2, respectively	527
Erwinia spp., *Pseudomonas* spp., *Xanthomonas* spp.	20 to 5 : 0 to 10	5 and 10	No or slight effect	221
Leuconostoc mesenteroides	21 to 2 : 0 to 40	10	No effect	115

ity maintained by some packaging material was a cause of increased mold spoilage in packaged tomatoes.[205] On minimally processed vegetables, atmosphere modifications tend to reduce growth of mesophilic bacteria and to increase growth of lactic acid bacteria[336] (Tables 25-17 and 25-18). Spoilage could be reduced by choosing the proper atmosphere composition. This was related to the inhibition of lactic acid bacteria on shredded or sliced carrots, but in other cases the relationship between spoilage reduction and microorganisms was unclear. The fate of foodborne pathogens on raw or minimally processed vegetables was unaffected by atmosphere modifications compatible with maintaining the quality of the product.[1,66,67,73,114,156,270,444]

In the US, modified atmosphere is used in combination with carbon monoxide to improve storage of fruits and vegetables during long distance shipment, with safeguards to prevent harmful effects on employees.[264,265] Carbon monoxide has fungistatic properties, which are enhanced under reduced oxygen concentrations and are more effective than the concentrations in carbon dioxide tolerated by the product.[177,418,420] Carbon monoxide concentrations of 5% to 10% with an oxygen concentration of 4% reduced growth of *B. cinerea* and soft rot of tomato.[267] However, carbon monoxide may hasten ripening of climacteric produce such as tomato.[177]

Anaerobic conditions permit development of strict anaerobes, either agents of spoilage or foodborne pathogens. Pectolytic clostridia caused soft rot of potato tubers in anaerobic conditions.[321] The risk of *Clostridium botulinum* development in minimally processed vegetables raised concern in the US about oxygen depletion in packages of minimally processed vegetables,[14] although the possibility that toxin may be produced before spoilage at temperatures below 10°C seems unlikely (Table 25-11, Section 25.4.3).

25.5.5 Use of the Physiology of Vegetables To Prevent Microbial Spoilage

Procedures based on the defense mechanisms of vegetable tissues have been developed to control microbial spoilage.[471] Some of these procedures rely on accelerating healing of wounds or lenticels by air drying at ambient or moderately high temperatures before storage, as in the cases of potato,[52,54,102] onion,[228,538] carrot,[300] and sweet potato and yam.[466] Fungi like *B. cinerea* frequently attack vegetables after colonization of senescent tissues such as the calyx of vegetable fruits. A treatment with plant growth regulators delayed senescence of aubergine calyx and reduced decay.[495] Selection of adequate practices during cultivation may increase resistance to microbial spoilage: reduction of nitrogen fertilization during production of roots reduced post-harvest bacterial spoilage of witloof chicory[297] and cultural practices may prevent cracking of tomato fruits and post-harvest decay.[394]

Strengthening of the cell walls of vegetables by calcium treatments, either applied directly on the

Table 25-17 Examples of the Effect of Controlled Atmosphere Storage on Fresh Vegetables. Effect of Atmosphere Modifications on Microbial Spoilage or Microbial Growth Are Given As Compared with Storage under Air

Vegetables	*Microorganisms*	*Temperatures °C*	*Atmospheres O_2 : CO_2 (%)*	*Spoilage or Microbial Growth*	*Reference*
Raw vegetables					
Tomatoes	*Erwinia carotovora*[a]	7 to 18	3 : 5	Spoilage reduction by >30% after 4 days	392
	Pseudomonas marginalis[a]	12.5	4 : 0 or 4 : 2 4 : 10 or 1 : 2	No effect Spoilage reduction, but softening of tomatoes	244
	Geotrichum candidum[a]	18.3	3 : 0 or 3 : 5 0.25 : 5	Spoilage increased No microbial spoilage but tomatoes injured	528
Aubergine	*Botrytis cinerea, Mucor mucedo*[a]	13	1.5 : 0 or 1.5 : 4	Spoilage reduction by 40% to 50% and by 20% to 15% for *B. cinerea* and *M. mucedo*, respectively, after 7 days	418
Celery	*Sclerotinia sclerotiorum, Botrytis cinerea*[a]	0 to 1	1.5 : 0 or 1.5 : 4	Spoilage reduction	419
Cabbage	Unspecified microbial spoilage	0	2.5 or 3 : 3 or 5	Spoilage reduction by 50%	404
	Botrytis cinerea and various molds and bacteria	0 to 2	3 : 5 to 6	Reduction of decay, to extents that varied among trials	205
	Alternaria tenuis, Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum	5 to 24	21 to 0.8 : 4.7	Reduction in spoilage for O_2 below 1.3%, at some temperatures	3
	Fusarium roseum			No effect or increase in spoilage by low O_2	
Asparagus	Aerobic mesophilic bacteria	4	15 : 6	Bacterial counts reduced by 1 log after 7 days	68
Cauliflower	Aerobic mesophilic bacteria	4	18 : 3	No effect on bacterial counts	68
Broccoli	Aerobic mesophilic bacteria	4	11 : 10	Bacterial counts reduced by 2 logs after 7 days	68
	Unspecified molds	4	3 to 1 : 0 to 9	Delay in spoilage for CO_2 over 3%, but CO_2 9% caused off-odors	56
Potatoes	Molds, mostly *Fusarium* spp.	8 to 10	2 : 1 to 12	Spoilage increased by CO_2 of 6% or more	417
	Various molds	8 to 10	2 : 6 or 12	Increased spoilage with molds normally weakly virulent	212

continues

Table 25-17 Continued

Vegetables	Microorganisms	Temperatures °C	Atmospheres $O_2 : CO_2$ (%)	Spoilage or Microbial Growth	Reference
Potatoes (continued)	*Erwinia carotovora*[a]	20	0 : 0	Increased soft rot	321
	E. carotovora[a]	10	0 : 0 1, 5 : 0, 16, 20	All atmospheres tested increased soft rot	323
	E. carotovora	10 and 16	10, 6, 2 : 0	Soft rot increased under 2% O_2	145
	E. carotovora[a]	22	0 : 0	Maximum number in potato tissues increased by 2 log cycles	145
Carrots	Molds, mainly *Sclerotinia*	0 to 1	3 : 0, 5, 10	85% to 100% carrots decayed, compared with 20% under air after 9 months	505
	Molds: mainly *Mycocentrospora acerina, Fusarium roseum, Phoma* spp., *Cylindrocarpon* spp.	0, 10, or 10.5	21 to 2.5 : 0	Incidence of decay increased by 2- to 4-fold for O_2 below 10%	26
	Unspecified microbial spoilage	1 to 2	21 to 13.5 : 0 to 7.5	Amount of decayed carrots increased with CO_2 content	526
Minimally processed vegetables					
Shredded lettuce	Numbers of aerobic mesophilic bacteria	4	21 to 3 : 0 to 10	No effect on bacterial growth, reduction of spoilage	47
Shredded carrots	Numbers of aerobic mesophilic bacteria and of *Leuconostoc mesenteroides*	10	21 to 0 : 0 to 40	No effect on total bacterial counts, growth of *L. mesenteroides* and spoilage only for CO_2>20% or O_2<2%	115
Shredded cabbage	Total bacterial counts	5	10 to 2.5 : 0 to 15	No effect on bacterial counts, reduction of spoilage under high CO_2	269

[a]Microorganism inoculated onto produce.

vegetable after harvest or applied as a fertilizer during cultivation in the field efficiently reduced decay of potato tuber by soft rot erwinias.[137,349,350] Extent of decay was negatively correlated with medullar or peel calcium content. Similar effects of calcium were obtained in the case of infection of cucumber fruit by *B. cinerea*.[127] Treatment of minimally processed carrots and courgette with calcium chloride reduced growth of aerobic bacteria.[251–253]

Infection induces defense reactions against spoilage microorganisms. This induction may be systemic as shown for carrot roots in which inoculation with *B. cinerea* and *S. sclerotiorum* near the root tip reduced infection after subsequent inoculation with these fungi near the crown.[354] Various treatments elicited defense reactions and increased resistance of vegetables to a subsequent inoculation with spoilage microorganisms[533]: Ultraviolet C radiation (wave length 180–280 nm) applied to a dose of 10 to 15 × 10^6 erg.cm^{-2} on carrots,[355,356] coating bell pepper with chitosan,[176] and application of glucan from fungal cell walls on bell pepper[7] caused phytoalexin accumulation, ultrastructural modifications, and an increased resistance to the fungal spoilage agent *B. ci-*

Table 25-18 Microbial Growth and Spoilage of Vegetables Packaged under Modified Atmospheres. Atmosphere compositions are presented as O_2 : CO_2 (%).

Vegetable	*Microorganisms or Populations*	*Temperature (°C)*	*Packaging Conditions*	*Atmospheres*	*Microbial Growth and Spoilage*	*Reference*
Raw vegetables						
Broccoli	Mesophilic and psychrotrophic bacteria, yeasts and molds		Shrinkwrapping Sealed packages with gas flushing	Close to air 9 to 4 : 1.4 to 5	Bacterial growth reduced by 1 log cycle and improved quality as compared with unpacked products	87
Broccoli	Bacteria, yeasts and molds	8	Packages sealed in air	3 : 8 at equilibrium	Reduction of microbial growth by 1 log cycle, as compared with unpacked products	364
Bell pepper	Mesophilic and psychrotrophic bacteria, yeasts and molds	13	Shrinkwrapping Seal packages with gas flushing	Close to air 18 to 10 : 0 to 3	Higher microbial populations but soft rot developed delayed as compared with unpacked products	88
Tomatoes	Unspecified spoilage microorganisms	15	Packages sealed in air	5 : 5 at equilibrium	Reduction of microbial spoilage as compared with unpacked products	376
Tomatoes	Mesophilic and psychrotrophic bacteria, yeasts and molds	133	Shrinkwrapping Sealed packages with gas flushing	20 to 11 : <2 20 to 5 : 1 to 10	Microbial growth increased by packaging, but spoilage delayed as compared with unpacked products	86
Minimally processed vegetables						
Shredded carrots	Total microbial counts, lactic acid bacteria	2, 6, or 10	Packages sealed in air with films of various permeabilities	6 to 2 : 20 to 60 after 12 days	Higher CO_2 increased growth of lactic acid bacteria and spoilage at 6° and 10°C	117
Sliced carrots	Total microbial counts, *Pseudomonas* spp., *Erwinia* spp., *Leuconostoc* spp.	4, 10, or 15	Packages sealed in air or vacuum packaging		*Erwinia* and *Pseudomonas* prevailed in air packaged products, *Leuconostoc* prevailed in vacuum packaged product at 10° and 15°C; spoilage increased in vacuum packages	100
Cut lettuce	Total microbial counts	8	Sealed packages with initial head space of air or 3 : 0	After 8 days, 2 : 14 or 0 : 10	No effect on microbial counts but reduction of spoilage	348

continues

Table 25-18 Continued

Vegetable	*Microorganisms or Populations*	*Temperature (°C)*	*Packaging Conditions*	*Atmospheres*	*Microbial Growth and Spoilage*	*Reference*
Shredded lettuce	Total microbial counts	2.8	Unsealed and sealed packages	19% CO_2 after 14 days in sealed packages	Lower total counts and improved quality in sealed packages	280
Shredded endive	Aerobic bacteria, lactic acid bacteria	3, 6, or 10	Sealed packages with initial head space of air or air + 20% CO_2	After 10 days, 10% to 17% CO_2 or 20% to 25% CO_2	Modification of head space reduced growth of aerobic bacteria, increased growth of lactic acid bacteria and reduced spoilage	107
Mixed salad	Total microbial counts	4.4	Sealed packages with initial head space of air or a 2.2 : 10 mixture	25% to 30% CO_2 after 14 days	Numbers of microorganisms similar in both conditions, but quality improved by modification of head space	405

nerea. Elicitation of defense reactions in the host tissues was also proposed as one mode of action of microbial antagonists (Section 25.5.3).

25.5.6 Resistant Cultivars

The resistance of vegetables to microbial spoilage differs between cultivars and may depend on susceptibility to penetration by the microorganism and its progression in the vegetable tissues or the production of inoculum (ie, fungal) from the lesion. Such differences have been shown for *Colletotrichum gloeosporioides* on pepper fruits,[333] *Colletotrichum lagenarium* on cucumber,[496] *Botrytis allii* on onion bulb,[306] *Rhizopus* spp. on sweet potatoes,[130] *Pseudomonas fluorescens* on broccoli heads,[106] *Erwinia carotovora* on potato tubers,[296] *E. carotovora* on carrot roots,[357] *Pseudomonas fluorescens* on lettuce,[361] *Phoma* spp. on potato tubers,[310] *Mycocentrospora acerina* on carrot roots,[299] and various spoilage agents on tomatoes.[79] In resistant cultivars, spoilage is usually only delayed and not prevented. Evaluation of resistance of cultivars may depend on testing procedures.[38] The validity of such tests for practical conditions must be considered carefully. Differences in the resistance to spoilage after minimal processing were found between different carrot cultivars,[32] but it is not clear whether they were due to specific traits of some cultivars or the result of growing and storage conditions.

Tomatoes transformed with an antisense polygalacturonase construct were more resistant to postharvest fungal spoilage.[290] The introduction of spontaneous mutation by traditional breeding gave similar resistance but was associated with defects in ripening.[290] The increased resistance of the transgenic tomatoes could reduce the cost of handling, storage, and transportation of vegetables or improve the quality of products with low added value that are not handled with great care, such as vegetables for canning. The defense reaction of vegetables could probably be increased by genetic transformation, but some antimicrobial compounds of vegetables may be detrimental to quality, as in the case of 6-methoxymellein, which causes bitterness of carrots,[472] or may be toxic to humans, as are furanocoumarins in carrot or in celery.[126,155,519]

25.5.7 Microbial Specifications

Microbiological criteria for minimally processed fresh vegetables have been published in European countries such as France, Germany, and the United Kingdom (UK).[319] In France, for example, limits at use-by-date for *Salmonella* (absence in 25 g), *E. coli* g^{-1} ($m = 100$, $M = 1{,}000$, $n = 5$, $c = 2$), and *L. monocytogenes* (100 g^{-1}) are legal standards.[13,15] Limits for aerobic mesophilic microorganisms and limits at production for *E. coli* ($m = 10$, $M = 100$, $n = 5$, $c = 2$) and *L. monocytogenes* (absence in 25 g) are only recommendations for manufacturers.[24] Such criteria are incorporated in codes of good manufacturing practices,[319] and their first purpose is to give indications of any deviations from these practices.[262] Aerobic mesophilic microorganisms are used as an indicator of quality, with the limitations described in Section 25.4.4, and limits have to be adapted to each type of vegetable.[24]

There is no microbial specification for raw vegetables, unless they are incorporated in a processed food, in which case they may have to meet standards defined for this food. Standards and inspection procedures for the quality of raw vegetables exist in several countries[11,266] and include the absence of microbial decay. Attempts have been made to predict the contamination of vegetables with spoilage agents, by methods such as detection of abnormal volatile metabolites produced by infected vegetables[388] or by measurement of incidence of microorganisms on the plant at harvest[232] but the authors of this chapter are not aware of any practical implementation of such measures.

25.6 MICROBIOLOGY OF PROCESSED VEGETABLES

25.6.1 Microbiology of Canned Vegetables

The frequency of contamination of vegetables with spores of *Clostridium botulinum* has been shown to range from 0% to 68% as a result of contamination of the field.[316,385] The minimum heat treatment to achieve commercial safety for foods with pH greater than 4.5 is a 10^{12} kill of *C. botulinum*; ie, a minimum of 3 minutes at 121°C (F_0 3 process). Survival of spores of *Clostridium botulinum* is the result of underprocessing and/or insufficient acidification. Some vegetables with a pH greater than 4.5, such as asparagus, cauliflower, or heart of artichokes are acidified to a pH lower than 4.5 and canned at a lower temperature to keep a firmer texture. Canned vegetables are often highly anaerobic. Oxidation-reduction potentials equivalent to between −440 mV and 0 mV at pH 7 have been reported.[367] The pH of most vegetables is between 4.5 and 7.0, with variations due to growing conditions or cultivars. The pH of tomato, for instance, ranges from 3.2 to 4.9[318]; canning of low-acid tomatoes with an inadequate heat treatment may lead to a botulism hazard.[163] Almost all vegetables at pH greater than 4.5 support growth of, and toxin production by, *C. botulinum.*[109,367,385] Vegetable and vegetable-based foods have been implicated in 24% of the recorded outbreaks of foodborne botulism in the world in the period 1950–1985[385] and in 51% of the outbreaks in the US in the period 1899–1977.[122] Most outbreaks were due to failures in home canning. An F_0 3 process does not totally eliminate other spore formers with heat resistance greater than that of *C. botulinum.* Anaerobic spore-formers that can cause spoilage include *C. sporogenes, C. butyricum, C. pasteurianum,* and thermophilic anaerobes, such as *C. thermosaccharolyticum* and *Desulfotomaculum nigrificans,* mainly in low-acid foods.[316] The aerobes, *Bacillus coagulans* in tomato products and the thermophilic species *B. stearothermophilus* in low-acid vegetables, cause a typical and fairly common spoilage known as flat-sour,[58,191] and have been detected in unprocessed vegetables.[302,539] In practice, therefore, the heat treatment used in canning vegetables to achieve the commercial stability is much greater than an F_0 3 process.[245]

Molds can have an influence on the canning process. Molds growing on fresh or heat-processed tomato can raise the pH (at least locally) to a pH that would allow growth of *C. botulinum.*[79,385] In contrast, vegetable matrices do not seem to markedly influence the heat resistance of bacterial spores during the canning process.[95,96,105,180,188,490]

In conclusion, contamination of canned vegetables with spoilage or pathogenic microorganisms is inevitable. Canning processes are therefore designed to give a 12-D kill of *C. botulinum* and to give commercial stability. Thus, safety and stability of canned vegetables depend quasi-exclusively on the control of the canning process and of the pH of the commodity, the other intrinsic characteristics of vegetables being of negligible significance.

25.6.2 Microbiology of Refrigerated, Processed Vegetables with Extended Shelf Life

Cooked vegetables are frequent ingredients in sous-vide and other refrigerated, processed foods of extended durability (REPFEDs). Cooking results in softening of vegetables.[414] The time to obtain optimal texture and taste ranges from 4 to 13 minutes at 100°C for root vegetables,[222] from 35 to 50 minutes at 90°C, from 4 to 12 minutes at 100°C, and from 3.6 to 4 minutes at 110°C for potatoes.[223,224] The heat treatment required to obtain cooked vegetables with an acceptable sensory quality is not high enough to kill spores of proteolytic strains of *C. botulinum.* That is the reason why REPFEDs must be kept under refrigeration temperatures. Failure in storage at low temperature of ready-to-eat and nonsterile cooked products can result in *C. botulinum* toxin production, as in sous-vide cooked potato[160,325] and has been implicated in numerous serious botulism outbreaks.[18,19,22,327,451]

In contrast to proteolytic *C. botulinum,* psychrotrophic nonproteolytic strains of *C. botulinum* will not survive the cooking process of vegetables because of insufficient heat resistance.[322] However, nonproteolytic *C. botulinum* can survive the pasteurization commonly applied to REPFEDs. In addition, vegetables (mainly brassicae and crucifers) can help heat-treated spores to recover.[489] Many cooked vegetables support growth of, and toxin production by, nonproteolytic *C. botulinum* at refrigeration temperature.[109,110] Time to toxin production is within the range reported for other foods. These are reasons why nonproteolytic *C. botulinum* should be considered as a potential hazard in cooked REPFEDs made of or containing vegetables.

25.6.3 Microbiology of Frozen Vegetables

Prior to freezing, most vegetables (with exceptions such as peppers and leeks) are subjected to a blanching process to inactivate enzymes that can cause deterioration of quality.[531] Blanching consists of exposure to hot water or steam at 90° to 100°C for a few minutes.[187,360,453] Examples of changes in numbers of microorganisms during blanching and freezing processes are shown in Table 25-19.

There are many changes in the numbers of microorganisms in vegetables during processing and freezing (Table 25-19). Washing vegetables has only a slight effect on the microbial contamination. In contrast, blanching has been shown to result in a 100- to 1,000-fold reduction in the microbial population. Procedures performed after blanching (cooling, conveying, packing) tend to increase the microbial load because of contamination from the equipment along the chain. The freezing in itself has generally no significant influence on the microbial population. In general, the numbers of microorganisms in frozen vegetables are stable or decrease slowly during storage at –9°C or –18°C.[360,475,499]

The numbers of microorganisms in frozen vegetables can range from below 10^2 cfu g^{-1} to above 10^7 cfu g^{-1},[44,213,360,423,475] usually from 10^4 to 10^6 cfu g^{-1}. Many different types of microorganisms have been

Table 25-19 Numbers of Microorganisms on Vegetables at Different Stages of the Blanching and Freezing Process

		Microbial Counts (log cfu g^{-1})				
Microbial Group	*Vegetable*	*Raw Material*	*After Washing*[a]	*After Blanching*	*Frozen Product*[a]	*Reference*
Mesophilic, aerobic bacteria	Spinach	6.6	5.5	3.0	4.5	360
		7.0	5.9	2.7	5.3	360
	Green beans	NT	5.8	2.7	6.0	412
	Southern peas	8.4	7.3	3.9	4.8	457
	Collard	7.0	6.4	4.0	4.6	455
		7.5	4.0	5.0	4.4	455
	Green beans	NT	6.3	3.1	6.0	479
		NT	5.6	3.6	4.7	479
	Green peas	NT	5.0	2.7	4.9	480
		NT	6.0	2.3	5.0	480
	Brussels sprouts	6.6	NT	1.8	3.0	499
Enterobacteriaceae	Spinach	4.9	3.0	<1.0	1.0	360
		6.1	5.0	<1.0	3.8	360
	Southern peas	5.3	4.2	2.6	2.5	457
	Collard	6.3	5.7	2.7	3.2	455
		6.5	2.9	3.2	2.9	455
Coliforms	Spinach	4.3	2.5	<1.0	1.0	360
		5.8	4.7	<1.0	3.7	360
	Green beans	NT	3.0[b]	1.3[b]	2.7[b]	412
	Peas	NT	2.4–3.7	<0–1.4	1.2–3.3	474
Coagulase-positive staphylococci	Green beans	NT	2.5[b]	1.3[b]	2.6[b]	412
Enterococci	Peas	NT	4.3–5.3	0.8–1.7	1.5–3.3	474
Yeasts	Southern peas	4.3	2.7	<1.0	2.3	457
	Collard	4.4	3.7	1.4	2.2	455
		4.8	0.5	2.4	1.6	455

[a] Use of antimicrobials for washing and time in a frozen state before enumeration generally not specified.
[b] log MPN.g^{-1}.
NT = Not tested.

isolated from frozen vegetables. These have been listed by Michels[360] and Senter et al.[457] The bacteria are among those found on fresh unprocessed vegetables, eg, *Enterobacter* spp., *Pseudomonas* spp., *Bacillus* spp., micrococci, and lactic acid bacteria. The incidence of yeasts and molds is generally lower than that of bacteria. A major part of the microflora originates from the processing equipment and depends on the hygienic practices used in the industry.

The incidence of *Salmonella* and *Staphylococci* in frozen vegetables is usually low.[44,360,476] *E. coli* was detected in 1% to 36.2% of the packs surveyed[44,213,478]; numbers were generally below 100 g^{-1}. Total coliform and fecal coliform counts gave a poor indication of contamination with *E. coli.*[44,478] *Listeria monocytogenes* was also detected in some frozen vegetables,[213,399] but not in others.[397] The presence of *L. monocytogenes* in frozen foods led to a recall of frozen vegetables.[20] Contamination of frozen vegetables with *L. monocytogenes* is also an indication of poor hygienic practices and/or insufficient blanching treatment. The recommendations on the microbiological limits for frozen vegetables proposed by the International Commission on Microbiological Specifications for Foods (ICMSF)[246] include only a limit on *E. coli*, with $n = 5$, $c = 2$, $m = 100$, and $M = 1{,}000$. The presence of *L. monocytogenes* in frozen vegetables is also undesirable and the French regulation, for instance, includes a limit of $m = M = 100$ *L. monocytogenes* g^{-1}, with $n = 5$ and $c = 2$ for all kinds of frozen vegetables.[21] The ICMSF[246] emphasized the commendable microbiological safety record of prepacked frozen vegetables.

25.6.4 Microbiology of Dehydrated Vegetables

As in freezing, the drying processes for vegetables include grading, washing, and blanching operations. The effects of blanching on vegetables to be dehydrated[359,485,511] are similar to those reported on vegetables to be frozen, ie, a marked reduction in the number of vegetative cells of microorganisms, but a less significant reduction in the number of spore-forming bacteria. In addition, vegetables subjected to drying are also sulfited to prevent browning and discoloration.[524] Despite the known antimicrobial effects of sulfites,[389] there is little information on their actual incidence in dried vegetables. Some vegetables, such as onion or garlic, are not blanched before drying.

Drying of vegetables is usually achieved with hot air. Because of the evaporation of water, the temperature of the product is generally below 50°C, while the temperature of hot air exceeds 80°C.[375] Commodities such as edible mushroom are dried at lower temperatures.[262] In the case of long exposure to drying, microbial growth can occur.[246,375,511] These could be the reasons why microbial counts after drying are not significantly different from those before drying.[359,485]

The number of microorganisms on dehydrated vegetables is very variable. Numbers of mesophilic, aerobic bacteria range from 10 to 10^8 cfu g^{-1}, with most counts between 10^4 and 10^6 cfu g^{-1} (Table 25-20). Numbers of coliforms, yeasts, and molds are also very variable (Table 25-20). The contamination originates from the raw material, from equipment along the processing line, and from storage. The diversity of type and species of bacteria found in dehydrated vegetables is as large as that on fresh unprocessed material (Table 25-21).

Enterobacteriaceae are the most commonly identified contaminants. Pseudomonads, although detected,[237] seem to have a lower incidence than on fresh unprocessed material. Blanching does not eliminate bacterial spores; *Bacillaceae*, thermophilic spore formers,[512] and sulfite-reducing clostridia have therefore been commonly observed (Tables 25-21 and 25-22). Significant counts of molds and yeasts are also reported (Table 25-20). Fungal contaminants are also very diverse and could be also found on unprocessed material (Table 25-21). A mycotoxin-producing *Aspergillus flavus* was detected in sun-dried African vegetables[5]; the authors did not discuss the risk to the consumer. Surveys of dehydrated vegetables for microorganisms of public health significance have shown the relatively low incidence of *S. aureus* and *C. perfringens*. In contrast, 25% of some samples contained *E. coli*, whereas others did not[274,435,458] and 5% of dried asparagus samples were contaminated with more than 100 *E. coli* g^{-1}. Salmonellae were detected in 0% and 23% of the samples analyzed (Table 25-20). The high proportion of positive samples was attributed to East Asian growing practices, including the use of human and animal wastes as fertilizers.[80] Despite this contamination, dehydrated vegetables do not appear to have been implicated in any outbreaks of salmonellosis.[246]

Many guidelines and limits on the level of microbial contamination have been proposed. The ICMSF,[246] for instance, recommends a limit only for *E. coli*, with $n = 5$, $c = 2$, $m = 10^2$, $M = 10^3$, whereas the French committee for the evaluation of the microbial quality of foods requires absence of *Salmonella* in 25 g and recommends *E. coli*, coagulase-positive staphylococci, *B. cereus*, and sulfite-reducing clostridia as indicators of hygienic standards. The proposed limits depend on the product (higher tolerance for mushroom for instance) and on its final use (lower tolerance for ready-to-use dehydrated vegetables such as potato flakes, than for products to be cooked, for instance).[262] Dehydrated vegetables are generally considered as safe, but they are potential sources of spoilage and pathogenic microorganisms when rehydrated or used as ingredients in high water activity food preparations. For example, dehydrated vegetables account for the major part of contamination of dehydrated soups.[273,292] For these reasons, the French legislation, for instance, has authorized the treatment of dehydrated vegetables with ionizing radiation at a maximal dose of 10 kGy to reduce their microbial contamination.[262]

25.7 CONCLUDING REMARKS

The development of minimally processed vegetables highlights microbiological issues linked to fresh vegetables. Elimination of microorganisms is not possible and growth or survival of most microbial pathogens is possible. Such products are therefore not intrinsically safe with regard to foodborne pathogens. Safety relies mostly on prevention of contamination from the field to processing, on a definition of a reasonable use-by date, and on refrigeration. Practices in the field or during harvest may be partly controlled by processors through contracts,

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Table 25-20 Numbers of Microorganisms on Dehydrated Vegetables

Microorganisms	*Dehydrated Vegetable*	*Samples Examined*	*Numbers (cfu g^{-1})*	*Reference*
Mesophilic, aerobic bacteria	Range	35	10 to 10^7	132
		110	10^2 to $> 10^6$, mostly 10^3 to $> 10^6$	237
		393	98 % $< 10^5$	274
		78	$<10^3$ to 10^6 , 83 % $< 10^5$	292
		139	< 10 to 10^7	511
	Onion powder and flakes	243	10^3 to 10^7	372
	Ground asparagus	89	10^2 to 10^8, mostly 10^3 to 10^7	435
	Potatoes	119	10 to 10^8, mostly 10^2 to 10^7	435
Coliforms	Range	35	< 10 to $> 10^6$, 83 % $< 10^3$	132
	Onion powder and flakes	243	< 1 to $> 10^3$	372
	Potatoes	340	< 1 to 10^5, 91 % $< 10^2$	435
	Ground asparagus	125	< 1 to 10^6, mostly 1 to 10^5	435
	Onion	146	10 to 10^6	512
Enterobacteriaceae	Range	110	< 10 to $< 10^5$, 90 % $< 10^3$	237
Enterococci	Range	35	< 10 to 10^6, 86 % $< 10^3$	132
Group D Streptococci	Onion powder and flakes	243	< 10 to 10^5	372
Yeasts	Potato sticks	40	98 % $< 10^2$	237
	Range	393	98.5% $< 10^3$	274
	Potatoes	114	< 1 to 10^6, mostly 1 to 10^6	435
	Ground asparagus	87	< 1 to 10^8, mostly 10^2 to 10^7	435
Molds	Potato sticks	40	98 % $< 10^2$	237
	Range	393	98.7% $< 10^3$	274
	Potatoes	113	< 1 to 10^3, 93 % $< 10^2$	435
	Ground asparagus	90	< 1 to 10^5, mostly 10^2 to 10^4	435
Yeasts and molds	Onion powder and flakes	243	10 to 10^4	372

although this is more difficult for imported vegetables, imports being necessary to provide products all around the year. In contrast, processors have little ability to control refrigeration after processing. This stresses the need for good practices for the purchase of raw material and for processing, and the need for legislation to minimize risks of contamination during cultivation and of temperature abuse during transport and retail. Contamination of vegetables in the field depends on agricultural practices but also on more general environmental issues such as pollution of water resources, and disposal of animal effluents and sewage treatment sludge.

Pressures for practices less damaging to the environment may lead toward a reduction in the use of antimicrobial chemicals in units processing vegetables, or may increase the cost of their use because of the need to treat effluents. Development of organic agriculture may influence the microbiology of vegetables, although heat generated during compost production should inactivate vegetative forms of bacterial pathogens.

The improvement of post-harvest techniques, such as the development of cold storage of some vegetables traditionally kept in the soil over winter, has reduced the incidence of some spoilage microorganisms while it increased others. Agricultural techniques also change dramatically, changing the causes of spoilage. For example, the need to produce tomatoes year round led to the development of glasshouse production, and nowadays in Europe fresh-market tomatoes are seldom grown in fields. Spoilage of tomatoes now depends mostly on the greenhouse microclimate. This situation may be modified again by the development of long-life tomato cultivars that permit long distance shipment at low cost from North African countries. Imports from remote countries may also reduce the need for long-term storage of some vegetables and this will presumably change the nature of microbial spoilage.

Table 25-21 Dominant Microorganisms on Dehydrated Vegetables

Microbial Group	*Dehydrated Vegetable*	*Dominant Organisms*[a]	*Reference*
Bacteria	Range	*Aerobacter, Escherichia, Bacillus, Achromobacter, Pseudomonas, Clostridium, Micrococcus, Lactobacillus, Leuconostoc*	511
Enterobacteriaceae	Range	*Klebsiella/Enterobacter* spp., *Proteus/Providencia* spp., *Citrobacter* spp., *Escherichia* spp.	237
Enterobacteriaceae	Onion	*Enterobacter aerogenes, E. cloacae, Citrobacter* spp.	458
Coliforms	Range	*Aerobacter* spp., *E. coli*	132
Enterococci	Range	*Streptococcus faecium, S. faecalis*	132
Aerobic mesophilic spore-forming bacteria	Onion	*Bacillus subtilis, B. licheniformis, B. cereus, B. pumilus*	458
Molds	African vegetables	*A. niger, A. flavus, A. fumigatus; Rhizopus oryzae, Penicillium oxalicum, Rhizomucor pusillus, Fusarium equiseti*	5
	Mushroom	*Mucor, Rhizopus, Penicillium, Aspergillus*	165
	Okra	*Aspergillus* spp., *Penicillium* spp.	175, 313
	Range	*Aspergillus, Mucor, Penicillium, Spicaria, Sporotrichum, Trichoderma, Herpocladiella, Alternaria, Fusarium, Monilia, Thamnidium*	511
Yeasts	Mushroom	*Pichia, Hansenula, Debaryomyces, Criptococcus, Rhodotorula*	165

[a] The names listed are those given in the referenced papers.

Table 25-22 Numbers of Pathogenic Bacteria on Dehydrated Vegetables

Bacterium	*Dehydrated Vegetable*	*Samples Examined*	*Prevalence (% of Samples) and/or Numbers (cfu g^{-1})*	*Reference*
Escherichia coli	Range	406	99.8% < 10^2	274
		78	86% < 10	292
	Onion powder and flakes	243	100% < 10	372
	Ground asparagus and potato	460	< 1 to 10^4, 97.8% < 10^2	435
	Onion	146	100% < 1	512
Salmonella	Range	68	2.9% positive (> 1 in 25 g)	80
		70	100% < 1 in 25 g	274
	Mushroom	162	6.8% positive (> 1 in 25 g)	80
	Asparagus	48	22.9% positive (> 1 in 25 g)	80
	Ground asparagus and potato	424	99.5% < 1 in 20 g	435
Staphylococcus aureus[a]	Range	70	100% < 10	274
		78	98.7% < 100	292
	Onion powder and flakes	243	100% < 10	372
	Ground asparagus and potato	166	100% < 10^2	435
Bacillus cereus	Potatoes	20	40% positive, 10^2 to 4×10^3	277
Clostridium perfringens[b]	Range	464	98.3% < 100	274
		78	97.5% < 2	292
	Onion powder and flakes	243	100% < 10^2	372
	Ground asparagus and potato	372	98.7% < 10^2, 100% <10^3	435

[a] Coagulase-positive Staphylococci.
[b] Sulfite-reducing Clostridia.

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26 Fermented and Acidified Plant Foods

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26.1 INTRODUCTION

The fermentation of foods of plant origin is of world economic importance. Fermented food products are made of various raw materials and by using microbial starters dominated by bacteria, yeasts, and/or molds. Several excellent books are available that introduce the reader into the area of fermented foods. These books range from general dictionaries[16,26] to edited volumes dealing with groups of fermented food commodities.[123,132,133,166] Indigenous or traditional fermented foods[152] form the basis of most industrialized food fermentation processes.[151]

This chapter discusses fermented foods made of vegetables, starchy cereals and roots, and proteinaceous seeds. Vegetable fermented foods include cabbages, olives, cucumbers, gherkins, onions, and others, that are preserved by a combination of salt, absence of oxygen, and acidity produced through fermentation mainly by lactic acid bacteria. A minority of yeasts also function in these products; they contribute to the preservation as well as the taste of the products. Certain vegetables, eg, gherkins, are also preserved without fermentation, but through the combination of salt, acid, and chemical preservatives. Some examples of such acidified vegetable products will be included.

Starchy cereals and roots are usually fermented without salt. A variety of processes will be illustrated, ranging from sour maize-based beverages fermented with lactic acid bacteria, to rye- and wheat-based sourdoughs in which yeasts play an essential role, and cassava fermentations aimed at detoxification of naturally occurring cyanogenic glycosides.

Proteinaceous leguminous seeds and oilseeds include peas, soya beans, groundnuts and others. The fermentation of this group of raw materials is targeted toward modification of the major chemical constituent, protein. This is achieved by molds in the preparation of tempe and soya sauce, for example, but proteolytic modification can also take place in bacterial fermentations in which *Bacillus* spp. dominate. Such partially degraded proteins include the much appreciated meat substitutes and condiments (flavorings).

26.2 VEGETABLES AND OLIVES

26.2.1 General Aspects

Range of Plant Materials Used and Products Produced

The traditional preservation of vegetables and oily fruits can be achieved by salting, either by immersing agricultural produce in watery salt solutions (brine), or by mixing fresh produce with crystalline sodium chloride. Because of the osmotic effect of salt, plant juices will ooze out of the vegetables. The nutrients in these juices enable microbial growth. If the storage takes place under anaerobic conditions, the combination of environmental conditions favors the dominance of lactic acid bacteria.[37,40,44,48,99,131,149]

Table 26-1 lists a variety of vegetables and oily fruits (especially olives) that can be fermented this way. The products obtained include sauerkraut, which became popular in the 17th century during long sea voyages, being a shelf-stable, excellent source of vitamin C. A rather similar product is Korean kimchi, obtained by lactic fermentation of salted vegetables, mainly Chinese cabbage, to which a variety of other vegetables, fruits, cereals, and even meat or seafood may be added. Other popular fermented products include pickles (fermented cucumbers) and olives, which are prized as salad ingredients. More recently, a wider variety of vegetables (see Table 26-1), as well as their juices, have been successfully fermented and enjoy increasing popularity as healthy snack and salad ingredients.

In addition to fermentation, a considerable proportion of some of these vegetables, particularly cucumbers, may be preserved by acidifying, salting, and pasteurization so that no fermentation or spoilage takes place, or by acidifying, adding preservatives, and cooling. These acidification procedures lead to a range of less expensive products, with a milder flavor and a more uniform quality.

Range of Types of Fermentation

Table 26-1 shows that lactic acid bacteria are the major fermenting organisms. Heterofermentative, followed by homofermentative lactic acid fermentation, results in both flavor and acidity. A relative minority group are fermentative yeasts. The latter have an important role to play, as scavengers of residual nutrients and oxygen. As a result, potential spoilage-causing microorganisms are inhibited.

Status

Table 26-2 lists some data on the production of fermented olives, cucumbers, and sauerkraut in the two major producing continents. Whereas the olive industry has its center of gravity in Europe (mainly Spain, Turkey, Greece, and Italy),[15] cucumber fermentation industry is mainly concentrated in the United States (US). Sauerkraut production is equally important in Europe (Germany and France) and the US.

Acidified pickles are popular in the United Kingdom (UK), the Netherlands, Germany, and the Scandinavian countries.

Composition of Starting Material

The chemical components found in vegetables used in fermentations, together with the main components of their microbial flora are listed in Table 26-3. Generally, vegetables have a high moisture content, providing a high water activity for microbial growth. Contents of protein, fat, and carbohydrates are relatively low (except for olives with a 20% w/w fat content). The carbohydrates consist mainly of reducing sugars and crude fiber. As the reducing (fermentable) sugars are located within the plant cell structure, they are not available for microorganisms in intact plant tissue. In order to enable fermentation, the cell structure needs to be disrupted, for instance, by mechanical damage as is done while shredding cabbage for sauerkraut. An alternative is to extract the sugars by an osmotic effect, which can be achieved using salt or brine.

26.2.2 Fermentation of Cabbage To Produce Sauerkraut

Method of Production

Sauerkraut (sour cabbage) is shredded and salted white cabbage (*Brassica oleracea*) that has undergone lactic acid fermentation. German food regulations require mininum 0.75% total acidity, pH < 4.1, 0.5%–3% NaCl, and less than 10% of total weight as strainable brine.[67] Special cabbage varieties for fermentation yield large, densely packed heads with a high dry matter content. A flow diagram for sauerkraut production is presented in Figure 26-1.

Green outer leaves and cores are removed and the cabbages are shredded into long strips, 1 to 2 mm wide. The cut cabbage is transported on a conveyor belt into tanks or silos. These may hold between 20 and 100 tons of shredded cabbage. Smaller plastic containers, holding 0.2 to 2 tons are also used. Salt (about 2% by cabbage weight) is added during conveying and filling. Even distribution of salt and controlled filling into containers are important for homogenous fermentation. Brine begins to form as

Table 26-1 Fermented and Acidified Foods Made of Vegetables and Olives

Raw Material	*Name and Origin of Fermented Product*	*Principle of Manufacturing Process*	*Characteristics and Use of Product*	*Predominant Microorganisms*	*References*
Fermented vegetables:					
Cabbage (*Brassica oleracea*)	Sauerkraut (Europe, US)	Wilt, remove outer leaves, wash, de-core, shred, mix with salt, ferment, sell fresh or preserve by refermenting, packaging, and/or pasteurization	Sour, preserved vegetable; winter food; excellent source of vitamin C	*Leuconostoc mesenteroides, Lactobacillus plantarum, L. brevis, Pediococcus* spp.	21, 40, 44
Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) with a variety of secondary ingredients	Kimchi (Korea)	Vegetable mixture (cabbage, radish, cucumber, onion, pepper, garlic, etc.), add approx. 5% salt, fill into earthenware vessels. Meat or fish may be added to flavor. Ferment 5 to 20 days at 10°–18°C.	Mildly acidic, carbonated vegetables, eaten as essential dish in Korea	*Leuconostoc mesenteroides, Enterococcus faecalis, Pediococcus* spp., *Lactobacillus plantarum, L. brevis*	16, 73
Cucumber (*Cucumis sativus*)	Pickles—Fermented cucumbers (US, Europe)	Grade, mix with brine, ferment, desalt, add herbs, pasteurize	Sour, refreshing side dish used as relish, snack, etc.	*Enterobacter, Lactobacillus plantarum, L. brevis, Pediococcus pentosaceus, Saccharomyces cerevisiae, Sacch. rosei*	21, 40, 44
Artichoke (*Cynara scolymus*), Carrot (*Daucus carota*), Celery (*Apium graveolens*), Tomato (*Lycopersicon esculentum*), Sweet pepper (*Capsicum annuum*), Beetroot (*Beta vulgaris*), Spring onion (*Allium cepa*)	Various pickles	Trim, peel, slice, add brine or solid salt, ferment, preserve (blanch, can, pasteurize)	Sour, refreshing snack	*Lactobacillus plantarum, L. sake, L. bavaricus*	14
Various vegetables	Lactic fermented juices (Europe)	Extract juice, pasteurize, inoculate with pure culture(s), ferment	Sour, refreshing beverages	*Lactobacillus acidophilus, L. bavaricus, L. bifidus, L. brevis, L. casei, L. delbrückii, L. helveticus, L. plantarum, L. salivarius, L. xylosus, Lactococcus lactis, Leuconostoc mesenteroides*	14
Various vegetables	Fermented salads (Europe)	Add pure thermophilic culture starter to sauce, add trimmed, cut vegetable, pack, ferment, cool	Refreshing mixed salads without conventional preservatives	*Lactobacillus acidophilus, L. plantarum*	131

continues

Table 26-1 Continued

Raw Material	Name and Origin of Fermented Product	Principle of Manufacturing Process	Characteristics and Use of Product	Predominant Microorganisms	References
Fermented olives:					
Olive (*Olea europaea*)	Treated green olives in brine (Europe)	Lye treatment, ferment in brine, pack	Snack, salad ingredient	*Lactobacillus plantarum, L. delbrückii*, fermentative yeasts (eg, *Hansenula anomala, Saccharomyces chevalieri*)	37, 48
Olive (*Olea europaea*)	Untreated natural black olives (Europe)	Ferment in brine, oxidize, pack	Snack, salad ingredient	*Saccharomyces oleaginosus, Hansenula anomala, Pediococcus, Leuconostoc, Lactobacillus* spp.	37, 48
Olive (*Olea europaea*)	Treated black olives in brine (Europe)	Ferment in brine, lye treatment and oxidation, neutralize, brine, can, and heat treat (botulinum cook)	Snack, salad ingredient	*Lactobacillus plantarum*	37, 48
Acidified products:					
Artichoke (*Cynara scolymus*), Carrot (*Daucus carota*), Celery (*Apium graveolens*), Cucumber (*Cucumis sativus*), Gherkins (*Cucumis anguria*), Beetroot (*Beta vulgaris*), Spring onion (*Allium cepa*), Sweet pepper (*Capsicum annuum*),Tomato (*Lycopersicon esculentum*)	Acidified pickles	Grade, wash, cut or whole, blanch, fill, add vinegar and herbs, cap, pasteurize	Sour, refreshing snack	Not fermented	44

Table 26-2 Production Data for Fermented Vegetables of Commercial Importance

Product	*Region*	*Production (tons)*	*Year*	*References*
Olives	World	893,000	1991–92	48
	Spain	227,000	1991–92	48
	Turkey	110,000	1991–92	48
	Italy	100,000	1991–92	48
	US	57,000	1992	44, 48
Cucumbers	Europe	45,000	1985	15
	US	590,000	1992	44, 48
Sauerkraut	Europe	220,000	1985	15
	US	170,000	1992	44, 48

soon as salt is added, as liquid is extracted from the cabbage by osmotic pressure.

Traditionally, no inoculation of cabbage is carried out. Starting with a well-cleaned silo, the microorganisms that occur naturally on the cabbage will grow and produce a succession of dominant microorganisms.

Silos and vats are covered at the top with a sheet of plastic, the edges of which are draped over the sides to form an open bag. Alternatively, a giant plastic bag with filling hole on top is placed on top of the shredded cabbage. Water, or a brine solution, is then pumped into the bag so that the cabbage is put under slight pressure, which helps to exclude the air, and soon the cut cabbage is immersed in its own brine, without exposure to air. The water used for the closure never comes into contact with the cabbage. In one or more corners of the fermentation silo, a perforated plastic pipe is inserted that reaches the bottom. This pipe allows the escape of gas produced during the first stages of fermentation. The evacuation of gas (CO_2) may also be enhanced by purging, ie, blowing controlled volumes of nitrogen gas or air, from the bottom upwards. The method of flexible sealing

Table 26-3 Chemical Composition and Main Components of the Microbial Flora of Some Vegetables Used in Fermentation

(per 100 g edible portion)		
Moisture (g)	85–95	
Crude protein (g)	0.5–5	
Crude fat (g)	<1	(olives: 20)
Fiber (g)	0.7–1.4	
Carbohydrates (g)	1–6	
Reducing sugars (g)	1–4	
Ash (g)	0.8–1.7	
Calcium (mg)	10–150	
Phosphorus (mg)	10–100	
Iron (mg)	0.5–5	
Sodium (mg)	5–100	
Potassium (mg)	250–700	
Manganese (mg)	500–800	
Vitamin A (I.U.)	—	
β-Carotene (mg)	<6	
Thiamine (mg)	0.02–0.15	
Riboflavin (mg)	0.03–0.30	
Niacin (mg)	0.02–0.25	
Ascorbic acid (mg)	10–100	

Epiphytic microflora ($\log_{10}N$ g^{-1}):	*Vegetables (range) (n = 99)*	*Cucumber (n = 44)*	*Cabbage (n = 44)*
Total aerobic bacteria	4–6	4.20	5.11
Enterobacteriaceae	3–3.5	3.59	3.59
Lactic acid bacteria	0.7–4	0.7	1.62
Yeasts and molds	0.3–4.6	0.2	<1

Source: Data from H.P. Fleming, K.H. Kyung, and F. Breidt, Vegetable Fermentations, in *Biotechnology, Vol. 9, Enzymes, Biomass, Food and Feed,* H.J. Rehm, G. Reed, A. Puhler and P. Stadler, eds., pp. 629–661, © 1995, VCH Verlag, and M.J.R. Nout and F.M. Rombouts, Fermentative Preservation of Plant Foods, *Journal of Applied Bacteriology,* Symposium Supplement, Vol. 73, pp. 136S–147S, © 1992, Blackwell Science, Ltd.

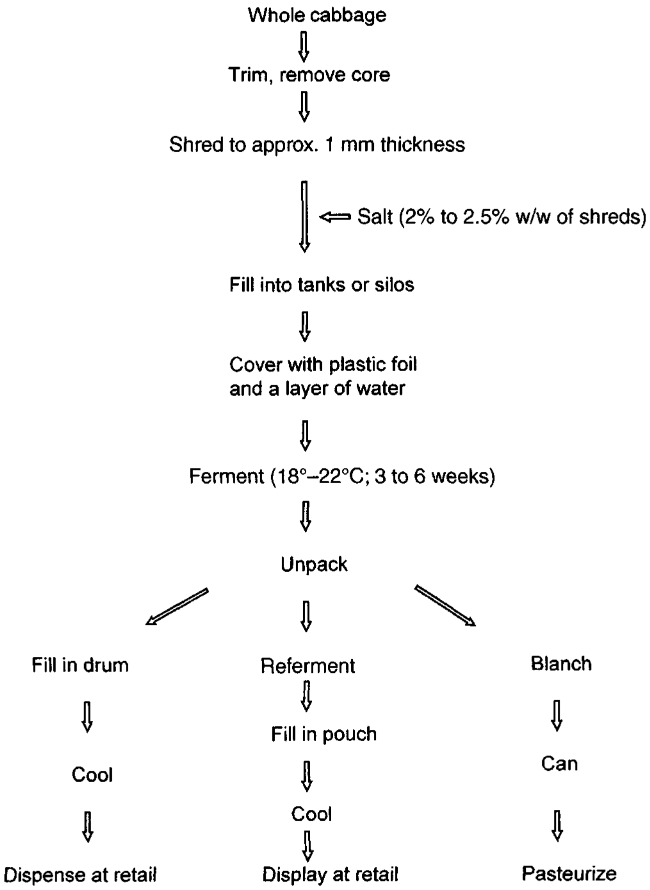

Figure 26-1 Sauerkraut Production Processes. *Source:* Data from O. Kandler et al., Microbial Interaction in Sauerkraut Fermentation, in *Proceedings, 4th International Symposium on Microbial Ecology,* pp. 302–308, © 1986, and J.R. Stamer, Lactic Acid Fermentation of Cabbage and Cucumbers, in *Biotechnology, Vol. 5, Food and Feed Production with Microorganisms,* G. Reed, ed., pp. 365–378, © 1981, Verlag Chemie.

provides optimum fit, in contrast to the ancient method of pressing the cabbage using wooden slats weighted down with stones.

Fermentation may be completed after several weeks at temperatures ranging from 15° to 20°C but may take several months in winter, when the temperature of unheated cabbage may be considerably lower.

The fermented product may be sold fresh from a retail barrel. In this case, the shelf life of the sauerkraut at refrigeration temperatures will be limited to 1 to 2 weeks. Alternatively, for supermarkets, it can be filled in sealed pouches and stored refrigerated. Before filling, the sauerkraut is refermented during an additional period to ensure that all residual sugars have been utilized. This refermentation is initiated by substituting part of the brine by water. These pouches have a shelf life of at least 2 months when stored below 7°C in the dark, or over 3 months when packed with carbon dioxide. If shelf-stable sauerkraut is required, the product must be blanched, canned, and pasteurized.

Microbiological and Chemical Changes during Fermentation

The natural flora of cabbage consists predominantly of strictly aerobic bacteria and some Enterobacteriaceae (Table 26-3). Lactic acid bacteria are always present but usually in numbers not exceeding 1% of total microflora.[44] However, even under conditions of Good Manufacturing Practice, shredder blades and conveyor belts transfer high numbers of lactic acid bacteria to the raw material.

Under conditions of tightly packed shreds, oxygen is rapidly depleted by respiring vegetable tissues and strictly aerobic bacteria.[121] At this stage, there is an increase in Enterobacteriaceae, which produce acids and cause an initial drop of pH to about 4.4 (Figure 26-2).

These Enterobacteriaceae are suppressed within a few days by the acids produced by fast-growing lactic acid bacteria. In this early stage of lactic acid fermentation, heterofermentative lactic acid bacteria, such as *Leuconostoc mesenteroides* and *Lactobacillus brevis,* predominate. Thereafter, homofermentative lactic acid bacteria take over, producing more acid and the pH may drop to 3.7 or lower. Figure 26-3 shows a succession of species that was recorded[119] in sauerkraut with 2.25% salt, fermented at 18°C.

Among the dominant homofermenters, *Lactobacillus plantarum* is a major representative, while *Pediococcus* spp. usually accounts for less than 10%. Other homofermenters, such as *Lactobacillus sake, Lactobacillus curvatus,* and *Lactobacillus bavaricus* have also been recorded, particularly in the early stages of fermentation.[67] Whereas the most spectacular events in microbial succession take place within a week at 18°C, fermentation continues for several weeks thereafter, during which time fermentable sugars are converted mainly to lactic acid and the pH drops steadily to below 3.5.[42]

Controlling factors in sauerkraut fermentation are temperature and salt concentration. At 18°C and 1% salt, heterofermentative lactic acid bacteria are favored, whereas at an impractically high temperature of 32°C and salt concentration of 3.5% homofermentatives are favored.[121] Normal conditions vary between 15°–22°C, with 2% to 3% salt (w/w on cabbage weight), with fermentation periods of 3 to 6 weeks.

The use of starter cultures has been considered, aimed at improving control over the process and the composition of the product. Sauerkraut contains

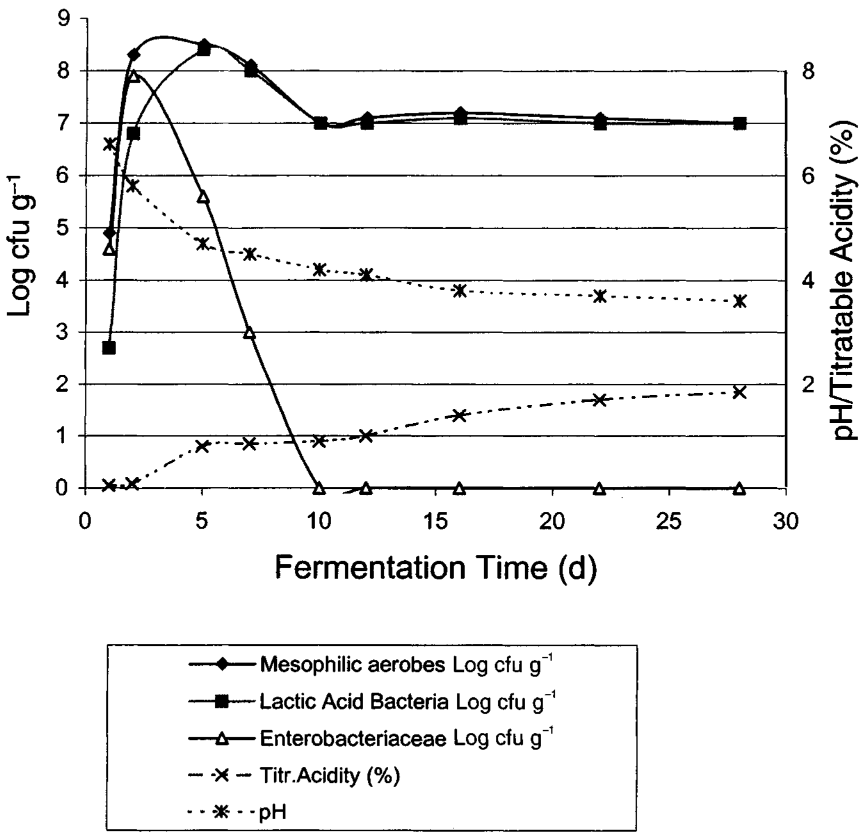

Figure 26-2 Microbiological and Acidity Changes during Sauerkraut Fermentation with 2% w/v NaCl at 18.3°C and Continuous Purging with N_2. *Source:* Reprinted with permission from H.P. Fleming, R.F. McFeeters, and E.G. Humphries, A Fermentor for Study of Sauerkraut Fermentation, *Biotechnology and Bioengineering*, Vol. 31, pp. 189–197, © 1988, John Wiley & Sons Inc.

D(−) and L(+) lactic acid, with an excess of D(−) lactic acid.[67] From a nutritional point of view, it is desirable to have as much L(+) lactic acid as possible, as this can easily be metabolized by the human body. *Lactobacillus bavaricus* produces exclusively the L(+) isomer, and using this organism as a starter culture the sauerkraut contained 70% to 90% of L(+) lactic acid. Addition of a homofermentative starter results in the early suppression of acid-sensitive *Leuconostoc mesenteroides*, which is, however, important for flavor production. Consequently, the product resulting from pure culture *L. bavaricus* inoculation has a rather bland flavor.

Another reason for using starter cultures is to minimize spoilage during storage. This will be discussed in the next section.

Table 26-4 shows the major chemical changes taking place during the fermentation. The data[42] refer to a "nitrogen-purged" sauerkraut fermentation as carried out under controlled conditions. The heterofermentative stage of sauerkraut fermentation is characterized by production of CO_2 (gas), lactic acid, acetic acid, and ethanol. However, because of purging, the CO_2 level was reduced quite soon after it has been produced. Acetylmethylcarbinol (acetoin), diacetyl, and 2,3 butylene glycol are by-products of fermentation that contribute to sauerkraut flavor. In addition, considerable amounts of fructose are reduced to mannitol by the heterofermenters. In Table 26-4 the fructose levels indicate excess sugar that has not (yet) been reduced. Gradually, all sugars that have leaked out of the plant tissue are converted into metabolites. At low pH, esters are formed spontaneously from fermentation products.

White cabbage may contain up to 4.5% w/w (250 mmoles l^{-1}) fermentable sugars, mainly glucose and fructose and some sucrose. These sugars gradually diffuse out of the shreds into the brine and are subsequently fermented. As a consequence the homofermentative stage may last for several weeks or months, during which these sugars are gradually converted to lactic acid (Table 26-4).

Prolonged fermentation can lead to a harshly acidic product, with 2% to 2.5% lactic acid. For this

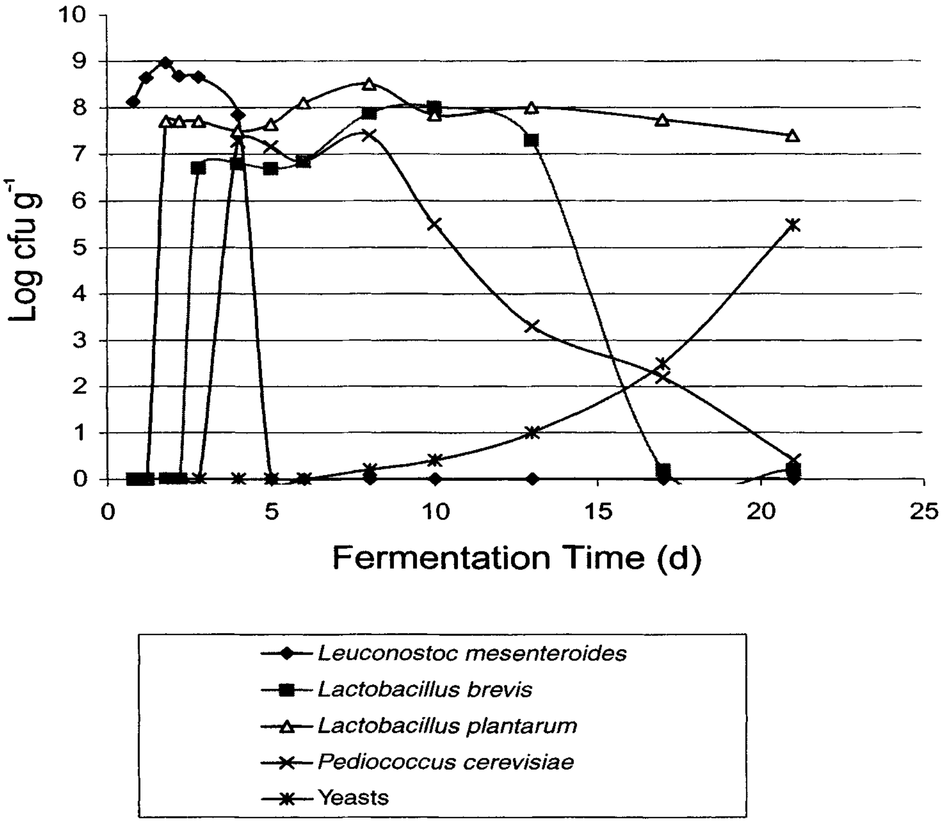

Figure 26-3 Succession of Species during a Sauerkraut Fermentation, with 2.25% w/v NaCl at 18°C. From day 1 to day 21 the pH fell from 4.8 to 3.5 and the titratable acidity increased from 0.15% to 1.78% expressed as lactic acid. *Source:* Data from C.S. Pederson, *Microbiology of Food Fermentations,* © 1971, AVI Publishing Co.

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reason the fermentation may be interrupted at an earlier stage, eg, when the concentration of lactic acid has reached 0.75%, which is the legal lower limit in Germany. This may be achieved by refrigeration, blanching, and/or pasteurization (see Figure 26-1). The product is then taken out, freshly packaged or canned, and sold as indicated in Figure 26-1.

Prevention of Spoilage

Several sauerkraut defects may be initiated during fermentation. These include:

1. Pink (sometimes gray) discolorations, especially when yeasts (*Saccharomyces exiguus* and pink yeasts) can develop, although *Lactobacillus brevis* has also been incriminated.[150] These form precursors that turn pink or gray when exposed to light and oxygen. A major cause of excessive yeast growth is inadequate acid production by the lactic acid bacteria, as a result of deficiency of manganese,[20] too high salt concentrations, too high temperature, or inadequate exclusion of air during fermentation.
2. Serious flavor defects are produced by butyric acid bacteria that develop and produce butyric acid. This type of spoilage may be because of use of soiled cabbage, irregular salt distribution, or slow development of lactic fermentation.
3. "Soft kraut" often occurs simultaneously with "pink kraut" as it is mainly caused by irregular salt distribution. If in some parts of the silo the salt level is too low, pectolytic enzymes are insufficiently inhibited. These enzymes may be produced by aerobic microflora, if air is inadequately excluded.
4. Slimy sauerkraut may result from polysaccharide (dextran) production by *Leucostoc* spp. when these bacteria grow out massively in fermentations carried out during winter time at unusually low temperatures.
5. Putrefaction occurs if aerobic microorganisms can develop; oxidation of organic acids results

Table 26-4 Chemical Changes Taking Place during the Sauerkraut Fermentation (2% w/v NaCl, 18.3°C, Continuously Purged with N_2)

Time (days)	*Glucose (mM)*	*Fructose (mM)*	*Sucrose (mM)*	*CO_2 (ml 100 ml^{-1} brine)*	*Lactic Acid (mM)*	*Mannitol (mM)*	*Acetic Acid (mM)*	*Ethanol (mM)*
0	35	30	1	25	0	0	0	0
1		29	2	20	0		0	20
2	48	30	3	30	1	35	1	30
3	70	26	2	60	40	50	30	35
5	72	8	1	145	85	80	65	40
7	78	0	0	20	95	95	80	40
12	73			32	120	95	85	40
16	60			10	160	95	85	40
22	40			30	200	95	90	45
28	30			30	230	95	82	35
60	2			25	245	95	90	60
150	1			20	270	95	90	65

Source: Reprinted with permission from H.P. Fleming, R.F. McFeeters, and E.G. Humphries, A Fermentor for Study of Sauerkraut Fermentation, *Biotechnology and Bioengineering*, Vol. 31, pp. 189–197, © 1988, John Wiley & Sons Inc.

in higher pH values, which in turn enable the growth of microorganisms, eg, pseudomonads.

Spoilage during fermentation may be controlled by adequate homogenous distribution of salt, exclusion of air, and adjusting salt concentration in relation to the temperature.

Exposure to oxygen during retail packaging of fresh sauerkraut should be kept at a minimum. Refermented sauerkraut (see Figure 26-1) is transferred by vacuum suction into plastic pouches, which are heat sealed and must be kept refrigerated (5°C) to avoid overgrowth by yeasts that survive fermentation or contaminate the product during postfermentation handling. Shelf life may be prolonged by adding potassium metabisulfite (if legally permitted), and sodium benzoate (max. 0.1% w/w); a shelf life of 8 to 12 months is feasible.[149] In the Netherlands, chemical preservatives are no longer used in sauerkraut, whereas refermentation is applied frequently.

During bulk storage of sauerkraut, extensive acidification may occur (see Figure 26-2 and Table 26-4). This results from homofermentative fermentation of available carbohydrates. If epiphytic homofermenters could be inhibited, eg, by enhancing heterolactic fermentation by *Leuconostoc mesenteroides,* the kraut would be stabilized. This has been achieved at an experimental scale, using a nisin-producing *Lactococcus lactis* strain,[52] in combination with a nisin-resistant *Leuconostoc mesenteroides.*[53] Nisin-paired starters[13] could suppress epiphytic homofermentative lactic acid bacteria, and postpone homolactic fermentation for at least 20 days, enabling a thorough heterolactic fermentation.

Control of Safety

Botulism arising from improperly canned vegetables has been well-documented.[41] In well-fermented sauerkraut, the combination of 2% NaCl and pH values below 4.5 prevents germination of *Clostridium botulinum* spores and toxin production.[92] However, defective sauerkraut (see above) may not fulfill these requirements, and requires special attention.

Another aspect of toxicity is the production of biogenic amines such as histamine, tyramine, putrescine, and others. In natural (uncontrolled) fermentations, up to 100 ppm histamine was recorded[14] with 200 ppm putrescine. Although these levels are not toxic, a reduction of biogenic amine accumulation could be achieved using selected pure cultures lacking decarboxylase activity.

26.2.3 Fermentation of Cucumbers

Method of Production

Cucumbers for pickling are produced from special varieties of *Cucumis sativis* L. and are harvested at a relatively immature stage, their value being inversely proportional to their size. Pickling cucumbers are preserved by three basic methods: fermentation (curing), pasteurization, and refrigeration. Figure 26-4 shows the principle of the process for fermentation; pasteurized and refrigerated pickles will be mentioned in Section 26.2.5 (acidified products).

Fermentation is the oldest method of cucumber processing. Washed and graded cucumbers are introduced into tanks with a capacity of 2.5 to 25 tons partly filled with a 10% sodium chloride solution (brine), so that during fermentation the concentration of salt may vary between 5% and 8% w/w.

Inoculation is not practiced commercially, but has been investigated at laboratory scale.[120] A microbial succession (see next section) takes place during the fermentation, starting with the microflora present on the cucumbers.[44]

Tanks are covered with headboards or protective plastic covers, such as those used in sauerkraut fermentation. Brines are periodically circulated for mixing. This mixing is also achieved when the tanks are periodically purged with air to remove dissolved CO_2 and prevent bloater formation (cucumbers with gas pockets inside). Originally this purging was done with nitrogen, but this is expensive and it was found that air could also be used. Toward the end of fermentation the salt concentration is gradually increased to between 8% and 16% w/w, so that the pickles may be stored for at least 1 year without spoilage (salt stock or brine stock).

Following fermentation and storage, excessive salt is removed by several changes of water. The pickles are filled into jars or cans, spices and condiments are added, and the product is pasteurized at an internal temperature of 74°C for 15 minutes.

Microbiological and Chemical Changes during Fermentation

A typical example of the succession of groups of microorganisms is shown in Figure 26-5. The rate and extent of growth are influenced strongly by the salt concentration in the brine. Figure 26-5 is relevant for a 40°Salometer brine. 100°Salometer refers to a 100% saturated NaCl solution; consequently, 40°S corresponds with 10.6% w/w NaCl in the brine. Because the initial salt concentration used in pickles is two to three times as high as in sauerkraut,[44] pickle fermentation is more typically dominated by salt-tolerant species. Initially, *Enterobacter* species may multiply and produce acids and gas (carbon dioxide and hydrogen). To prevent excessive gas formation the brine may initially be acidified with acetic acid to pH 4.5.

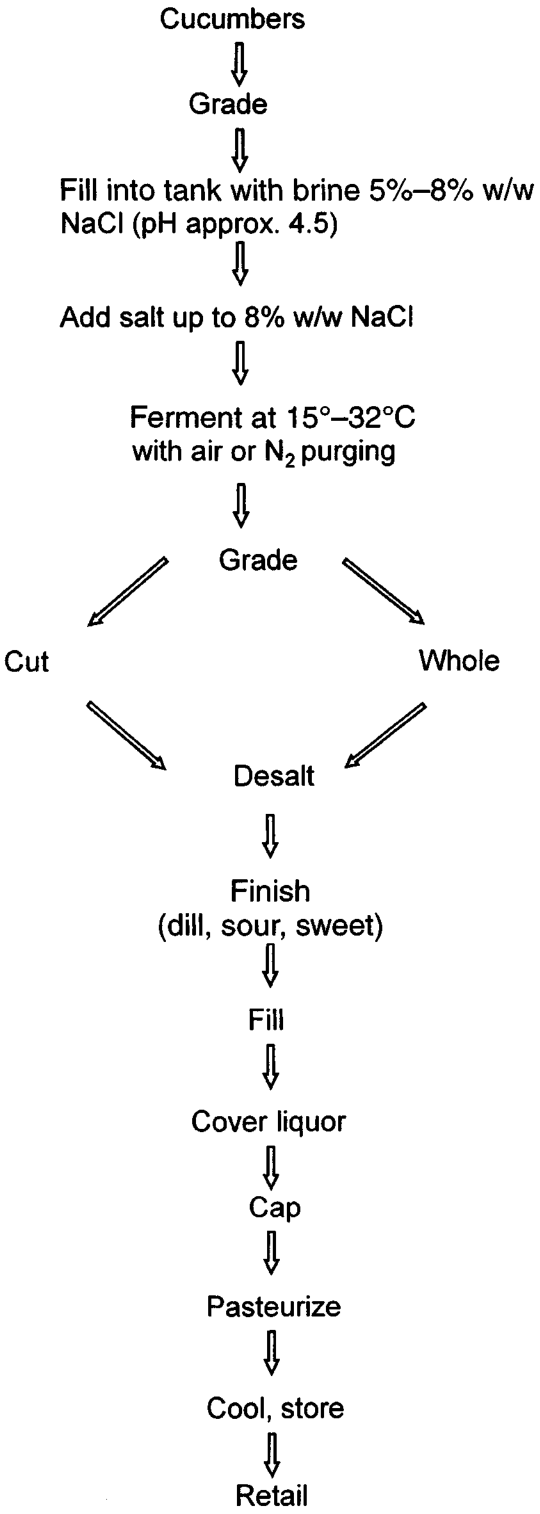
Cucumbers
Grade
Fill into tank with brine 5%–8% w/w NaCl (pH approx. 4.5)
Add salt up to 8% w/w NaCl
Ferment at 15°–32°C with air or N_2 purging
Grade
Cut
Whole
Desalt
Finish (dill, sour, sweet)
Fill
Cover liquor
Cap
Pasteurize
Cool, store
Retail

Figure 26-4 Cucumber Fermentation Process. *Source:* Adapted with permission from H.P. Fleming, K.H. Kyung, and F. Breidt, Vegetable Fermentations, in *Biotechnology, Vol. 9, Enzymes, Biomass, Food and Feed.* H.J. Rehm, G. Reed, A. Puhler, and P. Stadler, eds., pp. 629–661, © 1995, VCH Verlag.

Table 26-5 lists the major lactic acid bacteria and yeasts found in fermented cucumbers.

Due to its lower salt resistance, *Leuconostoc mesenteroides* is less important in the cucumber fermentation than in the production of sauerkraut. *Lactobacillus plantarum, L. brevis,* and *Pediococcus pentosaceus* are mainly responsible for acid production, and under favorable conditions (5% to 8% w/w NaCl; 21° to 27°C) fermentation is completed within 3 weeks. At higher salt concentrations the rate and extent of lactic acid fermentation are reduced and growth and gas production by yeasts is increased.

Both fermentative and oxidative yeasts have been isolated from brines.[44] The fermentative yeasts have the desirable effect of depleting all residual fermentable carbohydrate matter thus minimizing the growth of spoilage flora. This effect has been shown in a study on mixed *L. plantarum*-yeast (*Saccharomyces cerevisiae* and *Sacch. rosei*) starter cultures.[22] In contrast, oxidative yeasts are regarded as undesirable as they tend to oxidize organic acids and ethanol and to produce off-odors. Oxidative yeasts may be favored if air purging is applied, and fungi may develop and cause softening through their pectic enzymes; potassium sorbate (0.035% w/v) is sometimes added to the brine as an antifungal agent.

Cucumbers contain about 2% glucose and fructose as fermentable sugars which are converted to lactic and acetic acids, ethanol, mannitol, and CO_2. Figure 26-6 summarizes old, but still valuable, data[64] indicating the significant effect of brine concentration. With increasing NaCl concentration, less sugar is converted into acid. This corresponds with increasing gas production by fermentative yeasts that convert the sugar residues during the later stages of the fermentation process. Another fermentable substrate is malic acid (0.2% to 0.3%), which is degraded by *Lactobacillus plantarum* to lactic acid and CO_2. This conversion was discovered when *L. plantarum* was used as a starter and appeared to produce gas in pickle fermentation.[83,84]

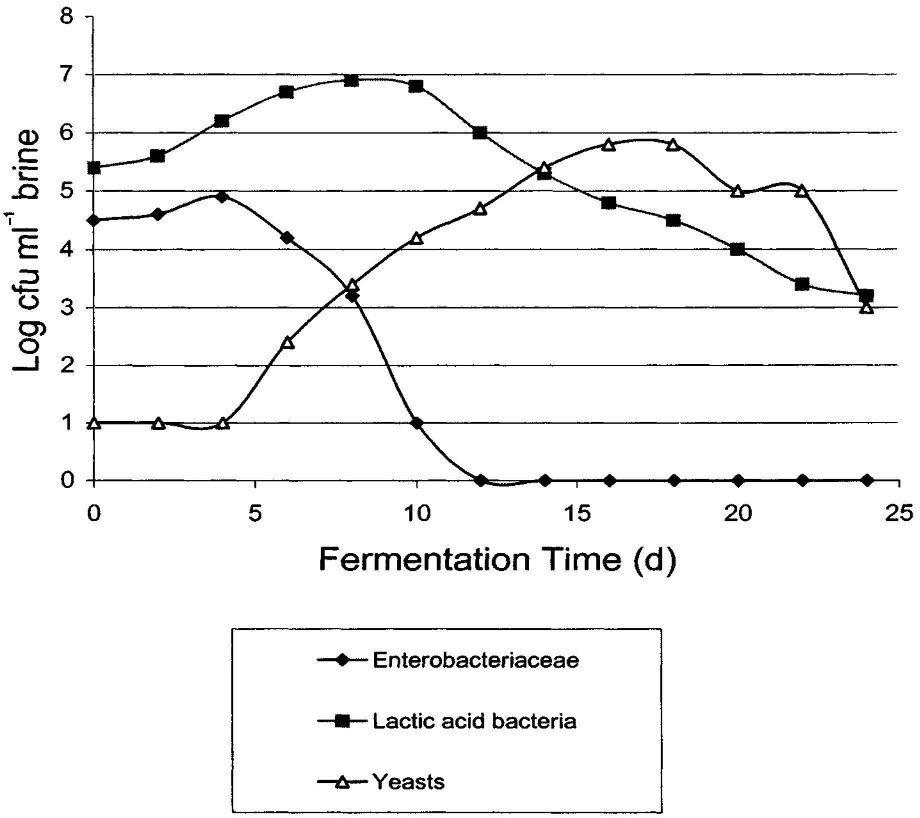

Figure 26-5 Microbiological Changes during Cucumber Fermentation. Initial brine concentration of 40° Salometer (10.6% w/w NaCl). *Source:* Data from J.L. Etchells and I.D. Jones, Bacteriological Changes in Cucumber Fermentation, *Food Industry,* Vol. 15, pp. 54–56, © 1943.

Table 26-5 Microflora of Fermented Cucumbers[40,44]

Lactic Acid Bacteria				*Yeasts*	
Genus and Species	*Fermentation Type*	*Main Product (molar ratio)*	*Configuration of Lactate*	*Fermentative*	*Oxidative*
Enterococcus faecalis	Obl.[a] Homofermentative	Lactate	L(+)	*Hansenula anomala*	*Candida krusei*
Lactococcus lactis	Obl. Homofermentative	Lactate	L(+)	*Hansenula subpelliculosa*	*Debaryomyces hansenii*
Leuconostoc mesenteroides	Obl. Heterofermentative	Lactate:Acetate:CO_2 (1:1:1)	D(–)	*Saccharomyces bailii*	*Pichia ohmeri*
Pediococcus pentosaceus	Obl. Homofermentative	Lactate	DL, L(+)	*Saccharomyces delbrückii*	*Rhodotorula* spp.
Lactobacillus brevis	Obl. Heterofermentative	Lactate:Acetate:CO_2 (1:1:1)	DL	*Saccharomyces rosei*	*Saccharomyces rouxii*
Lactobacillus plantarum	Fac.[b] Hetero-fermentative	Lactate	D(–), L(+), DL	*Torulopsis holmii*	
		Lactate:Acetate (1:1)	D(–), L(+), DL	*Torulopsis lactis-condensii*	
Lactobacillus bavaricus	Obl. Homofermentative	Lactate	L(+)	*Torulopsis versatilis*	

[a] Obligate.

[b] Facultative.

Source: Data from H.P. Fleming, Mixed Cultures in Vegetable Fermentations, in *Mixed Cultures in Biotechnology*, J.G. Zeikus and E.A. Johnson, eds., pp. 69–103, © 1991, McGraw-Hill, Inc.; and H.P. Fleming, K.H. Kyung, and F. Breidt, Vegetable Fermentations, in *Biotechnology, Vol. 9, Enzymes, Biomass, Food and Feed*, H.J. Rehm, G. Reed, A. Puhler and P. Stadler, eds., pp. 629–661, © 1995, VCH Verlag Weinheim.

Prevention of Spoilage

Cucumber fruits may retain their withered flowers, which are laden with pectolytic fungi and yeasts. If these microorganisms are transferred with the fruit into the brine, their pectic enzymes may cause pickle softening.[40] It is important therefore to minimize the introduction of cucumber flowers. Internal softening may also be the result of indigenous polygalacturonase,[44] the concentration of which increases in cucumbers with increase in size and maturity.

Another defect is the formation of bloaters or floaters, resulting from formation of CO_2 by lactic acid bacteria within the cavities. Bacteria, but not yeasts, are able to enter the fruit through stomata.[40] In this case, fermentation gases accumulate in cavities in the central part of larger cucumbers, bringing them to the surface of the brine. This quality defect is avoided by intermittent air purging, to remove dissolved carbon dioxide from the brine. However, purging with air introduces oxygen into the brine, and stimulates development of fungi and oxidative yeasts. As mentioned earlier, nitrogen has been used successfully for purging, but it is too expensive on a commercial scale.

In order to control oxidative fungi including yeasts, acetic acid or calcium acetate and potassium sorbate (less than 0.035%[44]) may be used. Acetic acid is effective against gas-forming enterobacters, fungi, and yeasts, whereas calcium ions act as firming agent on cucumber tissue by binding to pectic substances. Oxidative yeasts may also proliferate on the surface of brines in poorly covered tanks. These yeasts utilize lactic acid causing the pH to rise and thus allowing spoilage bacteria to grow. Butyric acid fermentation, resulting in offensive odors, may be initiated in brines with very low salt concentration, but this would be indicative of a gross processing failure.[43,44]

26.2.4 Fermentation of Olives

Method of Production

In 1991 and 1992 world production of table olives had reached 893,000 tons (Table 26-2), with the main production countries as follows: Spain (227,000 tons), Turkey (110,000 tons), Italy (100,000 tons), Morocco (85,000 tons), and Greece (80,000 tons).[48] Table olives are fruits of specific varieties of the cultivated olive tree (*Olea europaea sativa* Hoffmann et Link). They are harvested at various stages of ripeness (from green to black) and processed in different ways, to remove the natural bitterness caused by the glucoside oleuropein, and improve their organoleptic characteristics.[37]

Olives are fleshy stonefruits, the pulp accounting for 70% to 90% of the weight and the stone (endocarp) for 10% to 30%. The lipid content varies between 6%

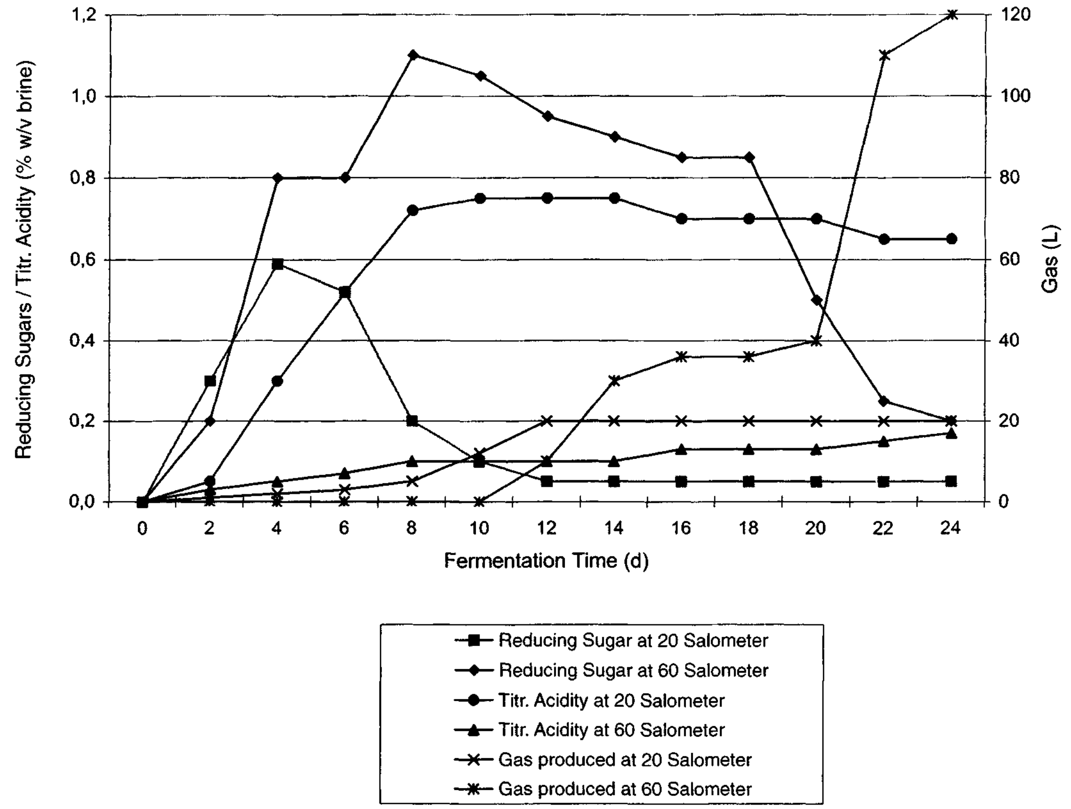

Figure 26-6 Chemical Changes Occurring during Cucumber Fermentation, As a Function of Brine Concentrations (20° and 60° Salometer, Corresponding with 5.3% and 15.9% w/w NaCl, Respectively). *Source:* Data from I.D. Jones and J.L. Etchells, Physical and Chemical Changes in Cucumber Fermentations, *Food Industry,* Vol. 15, pp. 62–64, © 1943.

and 30%. Fermentable sugars are glucose (1% to 3% of flesh), fructose (0.1% to 1.1%), and small amounts of sucrose and mannitol. Citric, oxalic, and malic acids are present in a total concentration of 0.5% to 1.0% of the weight of the flesh. This also contains 1% to 3% of phenolic compounds, mostly *ortho*-diphenols and their glucosides, which play an important role in fermentation because of their antimicrobial properties, specifically against lactic acid bacteria. The phenolic compounds are also important in the color development during ripening and in the darkening process for preparing ripe olives in brine.

The three major preparation methods for table olives are shown in Figure 26-7; major processing stages are summarized in Table 26-6.

In commercial production, olives are not inoculated with starter cultures. The processes rely on combinations of environmental factors (eg, salt, temperature, presence of microorganisms on fresh olives, and microflora attached on equipment).

For "treated green olives in brine"[48] the olives are picked when they have reached a green to straw-yellow color. They are treated in cylindrical or spherical polyester and glass fiber tanks with 1.3% to 2.6% w/v lye solution (NaOH) for 5 to 7 hours to hydrolyze oleuropein. The lye solution is then drained away and the olives are washed repeatedly with water, which removes some of the oleuropein and its hydrolysis products, other polyphenols, and some of the fermentable sugars. The olives are then put in brine containing 5% to 6% w/v NaCl to allow fermentation, which is mainly by lactic acid bacteria. The olives may be kept in the same brine until further processed, and sold after increasing the salt concentration to 8% w/v. These green olives, when fermented completely, are known as "Spanish-style green olives."

"Untreated natural black olives in brine" are prepared from violet to black olives. These are immediately brined (usually the brine has 8% to 10% w/v NaCl concentration initially) and fermented, predominantly by yeasts.

"Treated black olives in brine" (ripe olives) are prepared from olives, picked at various stages of ripeness, from yellowish-green to purple. They are usually stored in brine (5% to 7% w/v NaCl initially) prior to the oxidation (blackening) process, carried out with 1% to 2% w/v sodium hydroxide solutions and purging with air. After final washing, their pH may vary between 5.8 and 7.9. The black color is stabilized by adding iron salts. The olives are canned in a 2.5% to 5% w/v brine solution and sterilized.

Microbiological and Chemical Changes during Fermentation

"Treated green olives in brine" undergo the most extensive fermentation, in which lactic acid bacteria play a dominant role (Table 26-6 and Figure 26-8). Following lye treatment and washing, the olives are brined so that the final NaCl concentration is 5% to 6% w/v of brine during fermentation. In the first stage of fermentation (2 to 3 days) Gram-negative rods, Enterobacteriaceae (coliforms), and *Bacillus* spp. predominate. These are contaminants from olives, tanks, and washing waters. As a result the pH decreases from an initial value of 8.0 or higher, to 6.0 or below. In this stage gas formation (CO_2, H_2) is due to Enterobacteriaceae and *Aeromonas* spp. As fermentation progresses, *Pediococcus* and *Leuconostoc* spp. appear and a second stage of fermentation sets in, in which *Lactobacillus plantarum,* and to a lesser extent *Lactobacillus delbrückii,* take over. Figure 26-8[47] illustrates quantitative changes in predominant microorganisms, in combination with some chemical changes. During the second stage of fermentation (12 to 15 days), the pH should ideally drop to below 4.0. This pH drop depends on the concentration of fermentable sugars present and the buffering capacity due to salts of organic acids from olives, formed during treatment (so-called combined acidity or residual lye). Interestingly, unlike in sauerkraut and cucumber fermentation, in this type of olive fermentation pediococci may appear first, before leuconostocs and lactobacilli. This phenomenon may be due to the salt concentration and/or the resistance to polyphenols. During the third stage of fermentation, which lasts until substrates are exhausted, species of *Lactobacillus,* mainly *L. plantarum,* predominate and these coexist with a yeast flora.[38] *L. plantarum* requires vitamins and amino acids for its growth, and some of the yeasts appear to release B-vitamins.[134,135] The fermentative yeasts use a certain proportion of fermentable sugars, but their metabolites, such as ethanol, ethylacetate, and acetaldehyde contribute to the organoleptic characteristics of the product. Oxidative yeasts may occur also in superficial films. They should be kept low by anaerobiosis, as they oxidize lactic acid, raise the pH, and thereby may favor malodorous spoilage by *Clostridium* spp. Suppression of oxidative yeasts can be achieved by covering brine surfaces with plastic films. At the end of fermentation, the salt concentration is raised to 8% w/v to suppress possible development of *Propionibacterium,* which may cause an increase in pH and induce malodorous spoilage.

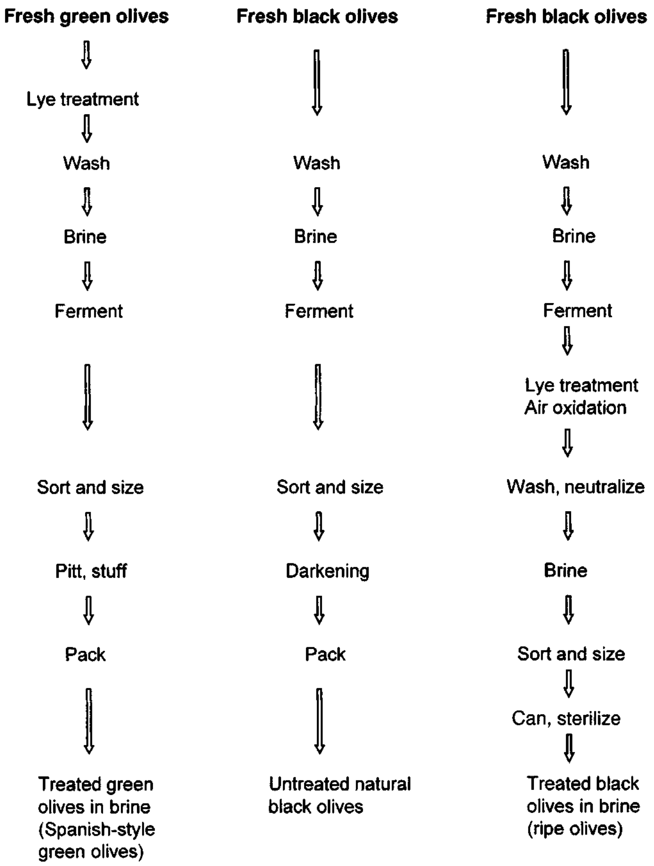

Figure 26-7 Three Methods of Olive Processing. *Source:* Adapted with permission from A. Garrido Fermandez, P.G. Garcia, and M.B. Balbuena, Olive Fermentations, in *Biotechnology, Vol. 9, Enzymes, Biomass, Food and Feed.* H.J. Rehm, G. Reed, A. Puhler, and P. Stadler, eds., pp. 593–627, © 1995, VCH Verlag.

"Untreated natural black olives in brine" are put immediately in brine of 8% to 10% salt (Table 26-6). The fermentation process is slow, as diffusion of soluble compounds through the skin of the fruits is slow when no lye treatment has been applied. Gram-negative bacteria are present in the first few days but disappear after 1 or 2 weeks. This fermentation is dominated by yeasts, which reach maximum num-

Table 26-6 Processing Stages and Characteristic Compositional Changes in Fermented Olives

Initial Phase			*Main Phase*			*Final Phase*			*General Characteristics of Final Product*
Process	*Changes*	*Microflora*	*Process*	*Changes*	*Microflora*	*Process*	*Changes*	*Microflora*	
Treated green olives in brine									
Lye treatment and washing	Hydrolysis of oleuropein; loss of sugars and organic acids; formation of organic acids from sugars	Aerobic epiphytic bacteria and molds; nonsporulating Gram-negative bacteria (Coli Aerogenes group); Gram-positive cocci (*Micrococcus, Pediococcus, Streptococcus, Leuconostoc*); some yeasts	Fermentation in brine (mainly lactic acid bacteria); secondary action of other microorganisms	Lactic acid formation; formation of other organic acids and volatiles; degradation of chlorophylls	*Lactobacillus plantarum; L. delbrückii*, fermentative yeasts (*Hansenula anomala, Candida krusei, Saccharomyces chevalieri, Candida parapsilosis, Hansenula subpelliculosa*)	Preservation in brine Bottling	None if no spoilage occurs	Spoilage microflora: oxidative yeasts, *Propionibacterium* spp., *Clostridium* spp.	Titratable acidity (TTA) approx. 1% as lactic acid; pH 3.6–4.2; NaCl 2%–8%
Untreated natural black olives									
None	None	Not relevant	Natural fermentation in brine; mainly yeasts; minority of lactic acid bacteria (LAB)	Slow loss of sugars, polyphenols, bitterness; formation of organic acids, ethanol, acetaldehyde, ethyl acetate	Major fermentative yeasts: *Saccharomyces oleaginosus, Hansenula anomala*; minority LAB: *Pediococcus, Leuconostoc, Lactobacillus* spp.	Preservation in brine Bottling	None if no spoilage occurs	Major oxidative yeasts: *Torulopsis candida, Debaryomyces hansenii, Candida diddensii, Pichia membranaefaciens*; propionic, butyric acid bacteria	TTA 0.3%–0.5% as lactic acid; pH 4.3–4.5; NaCl 6%–10%
Treated black olives in brine (ripe olives)									
Fermentation in brine by LAB and yeasts	Slow loss of sugars, polyphenols and bitterness; formation of organic acids, ethanol, and other volatiles	Coliform bacteria; *Lactobacillus plantarum;* of minor importance: *Pichia membranaefaciens, Pichia vinii*	Alkaline treatment; washing; oxidation by air; brining; bottling; heat sterilization	Hydrolysis of oleuropein; polymerization of polyphenols; loss of sugars and organic acids; organic acid formation from sugars	Either yeasts or lactic acid bacteria may dominate	None	None if no spoilage occurs	"Commercially sterile"[a]	pH 5.8–8.0; NaCl 1.5%

[a] See Chapter 3.

Source: Data from M.J. Fernandez Diez, Olives, in *Biotechnology, Vol. 5, Food and Feed Production with Microorganisms*, G. Reed, ed., pp. 379–397, © 1983, Verlag Chemie.

bers after 10 to 25 days. The rather high salt concentration compared with treated green olives in brine, is an important factor in the yeast dominance. Fermentative yeasts, such as *Saccharomyces oleaginosus* and *Hansenula anomala* are always found, along with some facultative oxidative yeasts, such as *Torulopsis candida, Debaryomyces hansenii, Candida diddensii,* and the oxidative species *Pichia membranaefaciens.*[79] However, *Pediococcus* and *Leuconostoc,* and in a later stage *Lactobacillus* species, also occur and produce acids. The major fermentation products are lactic and acetic acids, ethanol, ethylacetate, acetaldehyde, acetone, n-propanol, 2-methyl-propanol, and 2-methyl butanol. In this type of fermentation, air purging may be applied to remove excessive CO_2 and prevent gas pocket spoilage and shrivelling of fruits. As a consequence, the dissolved oxygen concentration is higher in this fermentation than in treated green olives in brine, and this favors growth of oxidative yeasts. The final pH is between 4.3 and 4.5, while the acid concentration varies between 0.3% and 0.5%. For this reason, at the end of fermentation the salt concentration should be raised to 8% to 10% to ensure adequate preservation.

"Treated black olives in brine" (ripe olives) typically undergo an alkaline oxidation process to achieve complete blackening of the fruit skin and uniform dark coloration of the pulp (Table 26-6). This oxidation process may be applied immediately after harvest, or the fruits may be stored in brine and fermented prior to oxidation. During storage in brine, air injection may also be applied with these olives to enhance the oxidation (blackening). Depending on varietal differences in phenol levels and salt concentration in the brine, either yeasts or lactic acid bacteria may dominate the fermentation. Induced lactic acid fermentation resulted in adequate preservation prior to oxidation treatment.[28] In order to cause blackening, fruits are repeatedly treated with sodium hydroxide solutions (1% to 2%) and water in which air is injected under pressure; usually this is achieved in three to five successive treatments. The alkaline oxidation of phenolic compounds causes a complete blackening of the fruit skin and a uniform darkening of the pulp. During finishing, most of the sodium hydroxide is removed; this is ensured by washing until the pH of the flesh is approximately 8. Finally, the olives are canned or bottled in a brine of 1.5% salt and sterilized.

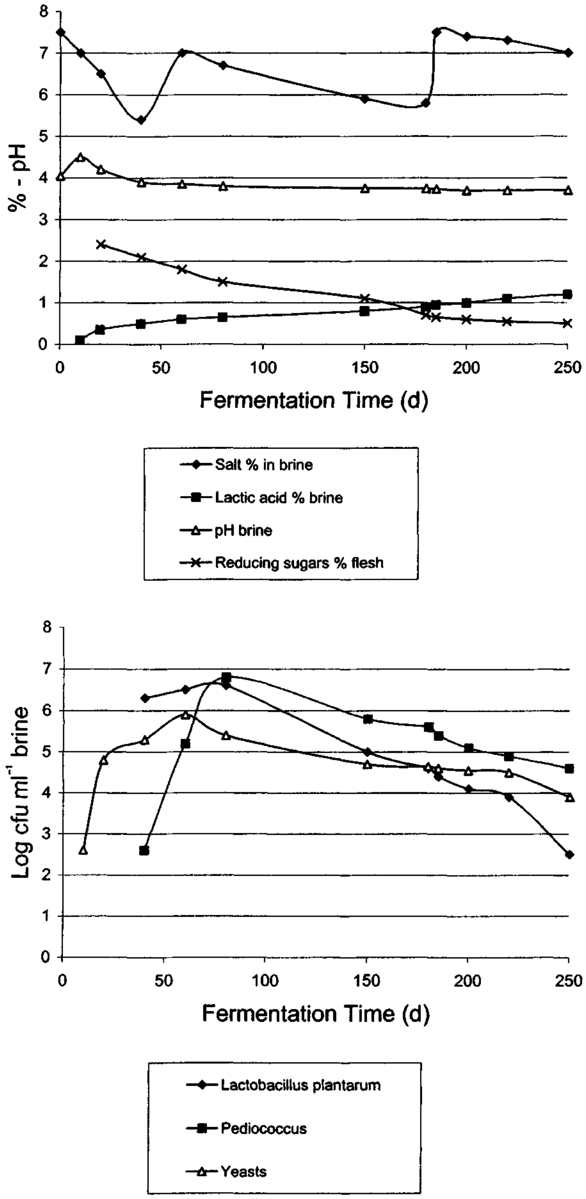

Figure 26-8 Chemical and Microbiological Changes during the Traditional Fermentation of "Treated Green Olives in Brine." *Source:* Data from P. Garcia Garcia et al., Lactic Fermentation during the Storage of "Alorena" Cultivar Untreated Green Table Olives, *Journal of Applied Bacteriology,* Vol. 73, pp. 324–330, © 1992, Blackwell Science, Ltd.

Prevention of Spoilage

In the fermentation of treated green olives in brine, initial proliferation of gas-forming Gram-negative rods may cause "aleunbrado," also called gassy spoilage or floaters. This type of spoilage is characterized by blisters, causing the skin to separate from the flesh of olives and also by the formation of gas pockets. The problem may be avoided by adjusting the initial pH to values around 6.0 or less by bubbling CO_2. Further pH reduction of brine pH to 4–4.5 is achieved usually by adding acetic, lactic, or hydrochloric acid.

In untreated natural black olives, gassy deterioration may be caused by yeasts, such as *Saccharomyces* and *Hansenula.* Also, *Bacillus polymyxa* and *B. macerans* may develop with Gram-negative bacteria early in fermentation and cause softening by their pectolytic enzymes. Certain pectolytic yeasts, such as *Saccharomyces kluyveri* and *S. oleaginosus,* may also cause severe softening in such directly brined olives.

In treated black olives in brine pasteurization, which is used occasionally prior to oxidative darkening, may prevent texture loss due to the pectolytic yeasts. This will also kill vegetative cells of *Bacillus* spp. It is not known whether remaining endospores of *Bacillus* spp. are able to germinate under the prevailing environmental conditions.

In all types of olives, malodorous types of spoilage may be caused by various anaerobic microorganisms. A putrid smell may be the result of growth of proteolytic *Clostridium* spp. Butyric acid spoilage may arise from development of butyric acid bacteria, such as *Clostridium butyricum.* Hydrogen sulfide spoilage results from *Desulfovibrio aestuarii.* Another off-odor called "zapatera" is characterized by the eventual development of a foul fecal-like stench, and is caused by *Clostridium sporogenes* and other clostridia in association with *Propionibacterium* spp. Contamination of brined olives with these anaerobic bacteria may come from water, dust, fermenters, or other equipment used in processing, or possibly from the surface of the olives. Spoilage usually starts at the bottom of fermenters where anaerobic conditions prevail. Malodorous fermentation can generally be prevented by working under good sanitary conditions and by controlling pH (≤ 4.6) and salt concentration ($\geq 5\%$).

Control of Safety

The most important safety issue in fermented olives appears to be the risk of *Clostridium botulinum* growth and toxin formation. Whereas anaerobic spoilage may occur during initial or later process stages, the occurrence of *C. botulinum* appears, however, to be rare.[76] A low pH (≤ 4.4), or relatively high salt concentration ($\geq 7\%$ w/v NaCl), or their combination is regarded as adequate protection against botulism.[92,95] Lactic acid bacteria will have produced lactic and acetic acid in addition to a reduced pH; this provides additional antimicrobial effects caused by the undissociated acids.[59,99] However, if such basic compositional requirements are not fulfilled, botulism cannot be ruled out completely. For instance, black olives were incriminated in a small outbreak of botulism of the B type[36]; the composition of the olives was insufficiently reported to evaluate their quality in relation with the above remarks.

A protective effect of lactic acid bacteria can be achieved by their production of bacteriocins, eg, plantaricin[136] and sakacin[143]; however, these were found to be ineffective against *C. botulinum.*[62,134]

Another aspect of food safety is the control of pathogens such as *Salmonella.* Investigations on the inhibitory effect of oleuropein showed that this glucoside does not inhibit *S. enteritidis,*[155] thus for safety one has to rely on other factors.

Spanish-style (green) olives, as they are produced commercially, eg, in glass jars or plastic pouches, are usually not pasteurized. Their pH is in the range of 3.5 to 4.0, with 0.5% to 0.75% w/v free acidity expressed as lactic acid and their salt concentration varies between 5% and 8% w/v. To spiced or stuffed olives, potassium sorbate may be added to a concentration of up to 0.05% w/v as sorbic acid. In pasteurized green olives the pH is 4 to 4.2 and the salt concentration about 5.5% w/v. Treated black olives in brine are sterilized as their pH varies in the range of 5 to 8 and their salt concentration from 2.5% to 5% w/v. Natural black olives cannot be sterilized for reasons of texture loss. For this reason, their pH in retail products is kept below 4.6 and their salt concentration at 7% to 8% w/v. In addition, potassium sorbate is allowed in this product to a final concentration of 0.05% w/v as sorbic acid.

Olives may carry mold propagules. *Aspergillus flavus* strains were isolated from natural black olives in brine, and their ability to produce aflatoxins was tested.[30] Under suitable conditions (aerobic incubation), mold growth was significant on fresh, damaged black olives and their paste, but was only weak on intact black olives. Only low levels of aflatoxins could be produced by strong aflatoxigenic strains. This implies that the use of intact olives for processing is preferable; furthermore, the olives must be protected from mold growth in hermetically sealed containers.

26.2.5 Acidified Products

Method of Production

Acidified products or vinegar-pickled vegetables are prepared (Figure 26-9) by pouring preboiled, hot vinegar onto the vegetables. These may be whole or cut cucumbers, gherkins, beets, olives, onions, peppers, or other mixed vegetables.[10,24] Salt, spices and herbs, herb extracts, sugar, and chemical preservatives are usually added. Prior to pasteurization, large pieces such as whole cucumbers, are blanched to exhaust air pockets. For unfermented pasteurized pickles, a liquor is used to fill jars and cans so that the final product contains 0.5% to 0.6% w/v acetic acid and up to 3% w/v salt, while the pH is about 3.7.[44] These products are shelf-stable for more than 1 year. Unpasteurized refrigerated pickles have a shelf life of not more than 3 months, during which time a slow lactic fermentation seems to occur. Their microbial safety status is rather unclear.

In Europe, unfermented sweet-sour pickles of gherkins and silverskin onions, for example, are quite popular. The liquor used to fill cans and jars may contain 2.5% to 4% w/v acetic acid, 5.5% to 8% w/v sucrose, and 2% to 2.5% w/v NaCl. With a liquor content of about 40% w/w, the concentrations in the final product would be 1% to 1.6% acetic acid, 2.2% to 3.2% sucrose, and 0.8% to 1.0% w/w NaCl, respectively. The products are pasteurized (30 minutes for 1 liter jars) at a waterbath temperature of 80° to 85°C. These products may be stored for more than 1 year at temperatures preferably not exceeding 15°C to prevent undesirable softening.

Microbiological and Chemical Changes during Production

In fresh pack pickles, survival of the pasteurization treatment by thermoresistant bacterial endospores may be expected; these would not be able to germinate in the acid product. In refrigerated pickles, some yeasts and lactobacilli might be present in the product.

Prevention of Spoilage

In fresh pack pickles, the preservation is achieved by a combination of low pH and pasteurization, and a relatively low concentration of acetic acid will suffice. For fresh pack cucumber pickles (Figure 26-9), the canning operation (glass jars or large 10 liter cans) is essentially the same as for fermented cucumbers, but the pasteurization is slightly more severe (15 minutes at an internal temperature of 78°C).

However, if nonpasteurized refrigerated pickles need to be preserved without preservative, the acetic acid concentration should be minimum 3.6% v/v[23] and the pH should be 3.5 or below. A preservative such as sodium benzoate (max. 0.1% w/w[149]) is usually added, and the product is stored at 1° to 5°C. Under marginal conditions, hardy species of yeasts (*Saccharomyces bailii* and *Pichia* spp.) and lactobacilli might grow and cause spoilage.[131,146]

It seems possible that acidified pasteurized vegetables may be prone to spoilage by *Alicyclobacillus acidoterrestris*, an acidophilic mesophile with a pH optimum between 3 and 4 and a minimum growth temperature of 26°C. This and more thermophilic species of *Alicyclobacillus* have been incriminated in spoilage of fruit juices, but so far occurrence in acidified pasteurized vegetables has not been recorded.[17,148,165]

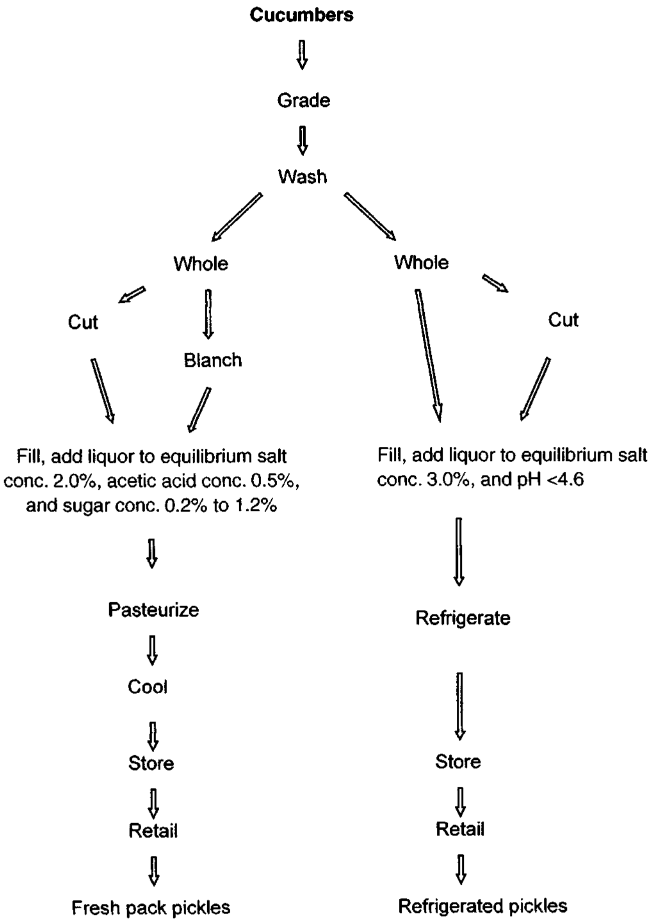
Cucumbers
Grade
Wash
Whole
Whole
Cut
Blanch
Cut
Fill, add liquor to equilibrium salt conc. 2.0%, acetic acid conc. 0.5%, and sugar conc. 0.2% to 1.2%
Fill, add liquor to equilibrium salt conc. 3.0%, and pH <4.6
Pasteurize
Refrigerate
Cool
Store
Store
Retail
Retail
Fresh pack pickles
Refrigerated pickles

Figure 26-9 Acidified Pickle Production Processes. *Source:* Adapted with permission from H.P. Fleming, K.H. Kyung, and F. Breidt, Vegetable Fermentations, in *Biotechnology, Vol. 9, Enzymes, Biomass, Food and Feed,* H.J. Rehm, G. Reed, A. Puhler, and P. Stadler, eds., pp. 629–661, © 1995, VCH Verlag.

Control of Safety

The safety of pasteurized acid foods depends on pasteurization and the fact that the pH (lower than 4.5) combined with the presence of organic acids, prevents the growth of pathogenic microorganisms. The safety of refrigerated pickles depends on the combination of refrigerated storage, a pH lower than 4.5, and the presence of organic acids, salt, and preservatives.

However, it is prudent to realize that these are not sterile products, and that surviving pathogenic microbes might proliferate when pickles are used at low concentrations as ingredients in neutral pH foods.

Next Page

26.3 CEREALS, TUBERS, AND ROOTS (STARCHY PLANT FOODS)

26.3.1 General Aspects

Range of Plant Materials Used and Products Produced

The most important starchy plant foods used in food fermentations are cereals, starchy tubers, and root crops. Table 26-7 provides an overview of starchy food plants that are processed into fermented foods for human consumption.

Cereals are grown worldwide, although a distinction can be made between tropical cereals (maize, millets, sorghum, rice) and those grown in moderate climates (wheat, rye, barley, oats).

Root crops and tubers are important suppliers of dietary starch. In root crops, the entire swollen root is edible, whereas tubers are swollen parts of the root system, or the stem. In both cases, the crop grows underground. The most important root crop is cassava, whereas sweet potato and Irish potato are major tuberous crops. Cassava is an extremely important staple food in tropical climates.

Range of Types of Fermentation

Table 26-7 summarizes a variety of fermented foods made from starchy food plants, their geographical origin, the principle of their manufacturing procedure, a description of the properties and utilization of the ready food, the microorganisms that dominate the fermentation, and references to the literature. By way of examples, products made of cereals (kenkey and sourdough) and of roots (gari) will be outlined in some detail in the following sections.

In order to obtain an edible fermented product from these food plants, a fermentation as well as a cooking step should take place. But in which sequence? In practice, both options are used.

In many "household-scale" fermentations in tropical countries, uncooked cereals and root crops are fermented first, and then cooked prior to consumption. Such products are listed under the first heading in Table 26-7: "cereals fermented prior to cooking," and "roots." In uncooked cereals and roots, endogenous enzyme activity generates adequate fermentable carbohydrates for a lactic acid fermentation to take place. Also, some starch-degrading bacteria and molds may contribute to the generation of fermentable carbohydrates from starch. Generally, lactic acid bacteria are poor degraders of starch. Consequently, it is essential for strong acidification that this enzymic amylolysis takes place. In many "household-scale" fermentations inoculation is not used. The minority of lactic acid bacteria on plant raw materials can achieve dominance at the conclusion of a natural succession of microorganisms. The climax population is formed by the most acid-tolerant, ie, lactic acid bacteria. Some examples of highly acid-tolerant species are *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Enterococcus faecalis*. A microbial succession takes time, and does not always produce the same result. A simple but very effective way of inoculation is the addition of previously fermented product to a new batch. This "back-slopping" approach causes the enrichment of high numbers of acid-tolerant lactic acid bacteria in the inoculum. Using back-slopping, lactic fermentation of cereals may be completed twice as rapidly as a fermentation without added inoculum. Cooking *after* fermentation and immediately before consumption inactivates any pathogenic bacteria, viruses, and parasites and increases the safety of the products. In many small-scale processes, hygiene is inadequate and in many tropical countries the quality of the water supply is not reliable.

Fermentation after brewing and cooking is required for the preparation of alcoholic beverages. In acidic conditions, fungi can develop well if their other environmental requirements are fulfilled. For mold growth, aerobic conditions are required, which explains the reason why lactic-fermented doughs become moldy on the surface if not protected adequately. On the other hand, when fermentable sugars are present, yeasts can develop in coexistence with lactic acid bacteria. This is the basis for a variety of traditional African beers made from maize, sorghum, or millets such as "busaa" and "pito."

Instead of using germinated grain (malt) to generate fermentable carbohydrates for brewing, mold enzymes are used in the saccharification of cooked rice for the preparation of Japanese rice wine (saké). The fermentation of saké consists of a mold solid-state phase, followed by a liquid fermentation dominated by yeasts and lactic acid bacteria.

In addition to these mixed fermented African and Asian alcoholic beverages, several popular European beers (Gueuze, Berliner white beer) are the result of inoculation with a combination of yeasts (*Saccharomyces*, *Brettanomyces* spp.) and lactic acid and other bacteria (*Lactobacillus*, *Pediococcus* spp.). The fermentation of some whiskey mashes is also of a mixed microbial character.

Fermentation after cooking the cereals or roots is used for lactic acid fermentations, and also for fer-

Table 26-7 Fermented Foods Made from Starchy Food Plants

Raw Material	*Name and Origin of Fermented Product*	*Principle of Manufacturing Process*	*Characteristics and Use of Product*	*Predominant Microorganisms*	*References*
Cereals fermented prior to cooking:					
Maize (*Zea mays*)	Mawè (Bénin)	Grind whole maize to grits, make dough by adding water, allow natural fermentation by LAB[a] and yeasts.	Sourdough, from which stiff porridges or dilute beverages are made (staple food).	*Lactobacillus fermentans, L. cellobiosus, Candida krusei*	58
Maize (*Zea mays*)	Uji (Kenya)	Dough maize grits with water, and allow natural lactic fermentation. Dilute and cook dough in boiling water to beverage of about 5%–7% dry matter.	Sour refreshing beverage, for breakfast, lunch, and snack. Also used as weaning food.	*Lactobacillus plantarum, L. fermentum, L. cellobiosus, L. büchneri, Pediococcus acidilactici, P. pentosaceus*	82
Maize (*Zea mays*)	Ogi (Nigeria)	Soak whole maize kernels in water; wet grind and lactic fermentation submerged in water; recover sour maize sediment and boil to thin beverage like Uji.	Same as uji.	*L. plantarum, L. confusus, L. murinus, L. agilis, Leuconostoc mesenteroides*	63
Maize (*Zea mays*)	Kenkey (Ghana)	Soak whole maize kernels in water; wet coarse grind, make stiff dough followed by lactic fermentation; cook half of the dough to gelatinized mass and mix with uncooked half; shape into balls, wrap in leaves, and cook submerged in water.	Massive, sour, bread-like maize product. In some areas, eaten 3 times daily (staple food).	Obligate heterofermentative lactobacilli, *Candida krusei, Saccharomyces cerevisiae*	50, 61
Sorghum (*Sorghum bicolor*)	Kisra (Ethiopia)	Sorghum flour mixed with water, inoculated with starter dough (previous batch), fermented, and baked.	Flat bread, used as starchy staple food.	*L. fermentum, L. reuteri, L. amylovorus, Candida krusei*	51
Wheat (*Triticum vulgare*) Rye (*Secale cereale*)	Sourdough (Europe, US)	Whole wheat/rye flour mixed with water, inoculated with previous batch of sourdough, fermented by mixed lactic acid bacteria and yeast populations.	Sour, stiff dough, used as leavening agent in the preparation of dough for sourdough bread preparation.	*L. plantarum, L. casei, L. büchneri, L. acidophilus, L. alimentarius, L. farciminis, L. delbrückii, L. fermentum, L. brevis, L. sanfrancisco*	137

continues

Table 26-7 Continued

Raw Material	*Name and Origin of Fermented Product*	*Principle of Manufacturing Process*	*Characteristics and Use of Product*	*Predominant Microorganisms*	*References*
Wheat (*Triticum vulgare*)	Tarhana (Turkey) Trahana (Greece)	Make dough with wheat flour, yogurt, bakers' yeast, tomato paste, onion, salt, peppers; ferment, dehydrate, grind, sieve.	Shelf-stable nutritious soup base.	*Saccharomyces cerevisiae, Streptococcus thermophilus, L. bulgaricus, L. lactis ssp. lactis, L. casei, L. lactis ssp. diacetylactis, Leuconostic cremoris*	60
Rice (*Oryza sativa*)	Idli (India)	Grind rice with bengal gram, add water, allow natural fermentation, prepare steamed cakes.	Steamed cake (staple food).	*Leuconostoc mesenteroides, Enterococcus faecalis,* yeasts	65, 66, 159
Rice (*Oryza sativa*)	Men (Vietnam) Ragi (Indonesia)	Make dough of uncooked rice flour, water, garlic, spices, and add previous batch; shape to small balls or tablets and allow to ferment and sun dry.	Used as fermentation starter for tapé, saké, and similar fermented rice products.	*Amylomyces rouxii, Endomycopsis fibuliger, Hyphopichia burtonii, Pediococcus* spp., *Lactobacillus* spp.	70, 96
Cereals fermented after brewing and cooking:					
Sorghum (*Sorghum bicolor*) Finger millet (*Eleusine coracana*)	Pombe (E. Africa) Busaa (Kenya)	Make dough with maize grits, allow natural fermentation by LAB and yeasts; fry soured dough on hotplate, mix gelatinized crumbs with ground germinated millet and water; brewing and fermentation by yeasts and LAB occur simultaneously.	Turbid, sour beer with considerable nutritional value.	*L. plantarum, Saccharomyces cerevisiae*	93
Maize (*Zea mays*) Sorghum (*S. bicolor*)	Pito (Ghana)	Wort is made from malted sorghum and water, a yeast-LAB starter (enrichment) is added, followed by alcoholic fermentation.	Turbid, sour beer.	Lactic acid bacteria, *Saccharomyces, Candida* spp.	25, 144
Rice (*Oryza sativa*)	Saké (Japan)	Steam-cook rice, inoculate with starter (ragi, men, or similar), ferment until fully liquefied, filter.	Rice wine.	*Aspergillus oryzae, Leuconostoc mesenteroides* var. *sake, L. sake, Saccharomyces cerevisiae*	168

continues

Table 26-7 Continued

Raw Material	*Name and Origin of Fermented Product*	*Principle of Manufacturing Process*	*Characteristics and Use of Product*	*Predominant Microorganisms*	*References*
Cereals fermented after cooking:					
Maize (*Zea mays)*	Maheu (S. Africa)	Cook maize grits in water, cool, inoculate with wheat bran and LAB (*L. delbrückii*), ferment at about 50°C	Refreshing sour beverage.	*L. delbrückii, L. bulgaricus*	57
Maize (*Zea mays)*	Togwa (Tanzania)	Suspend maize grits in water and cook to porridge; add milled germinated sorghum (malt) and some of previous batch of togwa; allow to ferment.	Refreshing sour beverage.	*L. lactis, Lactobacillus* spp., *Candida krusei*	68
Maize (*Zea mays*)	Pozol (Mexico)	Boil whole maize in lime water, dehull, grind to dough, shape into balls, wrap in leaves, allow lactic-fungal fermentation. Make pozol by suspending fermented ball in water.	Beverage.	*Leuconostoc, Lactobacillus, Candida* spp., *Trichosporon cutaneum, Geotrichum candidum*	106, 161
Glutinous rice (*Oryza sativa* var. *glutinosa*)	Tapé ketan (Indonesia)	Prepare steamed glutinous rice, sprinkle crumbled starter (see ragi) on it, cover, ferment.	Sweet, sour, and alcoholic rice snack.	*Amylomyces rouxii, Hyphopichia burtonii, Pediococcus* spp.	74
Rice (*Oryza sativa*)	Risogurt (Korea)	Extrude rice flour, add soyabean protein isolate, ferment with LAB to yogurt-like product.	Refreshing and nutritious sour beverage.	*Streptococcus thermophilus, Lactobacillus bulgaricus*	160
Roots:					
Cassava (*Manihot utilissima*)	Gari (Nigeria)	Wash, peel, grate cassava to pulp; press pulp in woven bag, allow to drain juice and ferment; roast fermented crumbs to dry, gelatinize starch, and remove HCN.	Instant starch food, eg, for breakfast; shelf-stable staple food.	*Leuconostoc, Enterococcus, Lactobacillus, Geotrichum candidum*	116, 164
Cassava (*M. utilissima*)	Kokonte (Ghana)	Wash, peel, cut cassava, cover, allow solid-substrate fermentation; pound and cook.	Stiff cooked dough; staple food.	Lactic acid bacteria, molds (*Rhizopus, Neurospora* spp.)	78
Cassava (*M. utilissima*)	Attiéké (Ivory Coast)	As gari, but steam cooked after fermentation.	Ready-to-consume cooked crumbs; staple food; not shelf stable.	See gari	39
Cassava (*M. utilissima*)	Farinha de Manioca (Brazil)	Wash, peel, sour fermented starch, sun dried.	Flour used to cook stiff pasty dishes.	See gari	27

continues

Table 26-7 Continued

Raw Material	Name and Origin of Fermented Product	Principle of Manufacturing Process	Characteristics and Use of Product	Predominant Microorganisms	References
Cassava (*M. utilissima*)	Lafun (Nigeria)	Wash, peel, cut, ferment submerged in water; drain water and sun dry fermented pieces; grind to flour.	Flour used to cook stiff pasty dishes (eba) (staple food).	*Bacillus* spp., *Corynebacterium manihot, Candida* spp., *Geotrichum candidum, Klebsiella* spp., *Leuconostoc mesenteroides, Lactobacillus plantarum, Micrococcus luteus*	3, 115
Cassava (*M. utilissima*)	Fu-Fu (Nigeria)	Wash, peel, cut in half, ferment submerged in water; drain water and grate roots, ferment again, sieve to remove fiber, dewater, cook, pound.	Cooked, pounded mash eaten as starchy staple food.	*Bacillus, Candida, Corynebacterium, Lactobacillus acidophilus, L. casei, L. fermentum, L. brevis, L. delbrückii, L. sake, Leuconostoc, Klebsiella* spp.	116, 164
Tubers:					
Sweet potato (*Ipomea batatas*)	Tapé ketella (Indonesia)	Peel, cut, steam cook, inoculate with ragi as in tapé ketan	Similar to tapé ketan (above)	See tapé ketan	70

[a] LAB = lactic acid bacteria.

mentations with mixed fungal starters (eg, tapé ketan and tapé ketella). Important consequences of cooking first are: (1) the gelatinization of the starch causes a considerable increase of viscosity, which makes the product more difficult to handle, (2) the endogenous enzymes are inactivated and thus, no auto-amylolysis will occur, and (3) the epiphytic microorganisms are killed. Consequently, if a fermentation must follow, a source of fermentable carbohydrates and the right type of functional microflora must be provided. In Togwa, for example, this is achieved by adding germinated sorghum (malt) to the cooked maize. The enzyme activity of the malt will generate maltose from the maize starch; moreover, sorghum malt contains high levels of bacteria and yeasts, and thus it provides a mixed inoculum. However, the quality of this inoculum leaves much to be desired from the points of view of safety and product quality. In Maheu, a more controlled process is achieved. Addition of wheat bran (containing cereal enzymes) ensures generation of fermentable carbohydrates, whereas the fermentation takes place with a selected thermophilic lactic acid bacterium at about 50°C. In practice, this temperature is selective for *Lactobacillus delbrückii* and is too high for enteropathogenic bacteria to survive. A similarly controlled process is that of risogurt, in which rice is cooked to gelatinize, partly degraded using amylolytic enzymes, sterilized by autoclaving, and fermented with a defined, mixed culture.

Status

Fermented cereal and root products are known and consumed worldwide. They are often used as staple foods in tropical countries. Production may be on a household scale and sales may serve as a source of (additional) income to the family. Alternatively, some products (such as uji, sourdough, pombe, saké, maheu, and gari) are also processed on an industrial scale facilitating some control of microbiological and chemical changes, and of the microbiological safety of the products.

Composition of Starting Material

Ranges of chemical components of starchy foods are listed in Table 26-8. Cereals contain 25% to 30% moisture at harvest, and drying to 10% to 14% moisture content makes them rather stable for storage. Cereals are seeds and consist of an embryo, an endosperm, and multiple protective layers referred to as "bran." Whereas the embryo has a relatively high lipid and protein content, the endosperm contains the reserve nutrients mainly in polymeric, water-insoluble form (starch, protein, lipids). The bran has a rather high cellulose and hemicellulose content, and significant levels of silicate and minerals (ash). Other important compounds in cereal endosperm and bran are phytic acid (inositol hexa phosphate) and tannins (condensed polyphenols). Both substances can form chemical complexes with minerals, starch, and proteins; as a result, they have a negative effect on a number of important processes. First, they reduce the bioavailability of minerals in the digestive tract; as such they are regarded as "antinutritional factors (ANFs)." Moreover, the tendency of phytic acid and tannins to bind with proteins also causes inhibition of enzymes and metabolic activities of microorganisms. During the germination of seeds, a variety of saccharolytic, proteolytic, and other enzymes are activated and/or produced mainly from within the aleurone layer of the bran. The result is that the endosperm material is "modified," ie, polymeric material is degraded to oligomers and further to maltose, small peptides, and free amino acids, for example. During germination, ANFs such as phytic acid are degraded. This enzymatic degradation also takes place in grains that have been ground to a meal that is mixed with water. Obviously, this type of bioconversion can only take place in cereals that have not previously been heated.

Roots and tubers have a much higher moisture content and contain considerably less protein than cereals. Unlike cereals, they are highly susceptible to spoilage. Bitter varieties of cassava contain toxic glycosides. Particularly in Africa, these bitter varieties are widely preferred by farmers as they are much less affected by wild animals (monkey, bush pig) and theft. The toxic glycosides (linamarin, lotaustralin) can be degraded to glucose, acetone, and the highly toxic HCN if they are brought into contact with the enzyme linamarinase. In the intact root, the cell membrane–bound enzyme is physically separated from linamarin. However, accidental or deliberate damage to the root tissue starts the enzymatic process. This is important during food processes aimed at detoxifying bitter cassava.

Cereals, roots, and tubers carry a mixed soilborne and airborne microflora, characterized by the presence of bacteria (eg, *Enterobacter aerogenes*, *Erwinia* spp., *Bacillus mesentericus*, and *Flavobacterium* spp.), yeasts (eg, *Cryptococcus albidus*), and molds (*Fusarium*, *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* spp.). Lactic acid bacteria are present but in very low numbers. Much of this original flora is removed during preparatory processing, for example, by peeling, dehulling, and washing.

Table 26-8 Chemical Composition and Main Components of the Microbial Flora of Some Starchy Food Plants Used in Fermentation

(per 100 g edible portion)	*Cereals*	*Roots and Tubers*
Moisture (g)	8–13	50–80
Crude protein (g)	5–14	0.2–2
Crude fat (g)	1–5	<0.4
Fiber (g)	0.6–1.8	0.8
Carbohydrates (g)	62–80	17–37
Reducing sugars (g)	0.5–2.5	0.3
Ash (g)	0.8–1.9	1.1
Calcium (mg)	10–350	7–150
Phosphorus (mg)	90–400	60
Iron (mg)	0.5–5	0.5–2
Sodium (mg)	5–10	10
Potassium (mg)	100–400	600
Vitamin A (I.U.)		0–100
Thiamine (mg)	0.1–0.6	0.1–0.15
Riboflavin (mg)	0.05–0.15	<0.06
Niacin (mg)	1–3.5	0.3–1
Ascorbic acid (mg)		<20
Epiphytic microflora ($\log_{10}$ N g^{-1}):		
Bacteria:		
Aerobes	4.5	4–6
Enterobacteriaceae	3.5–5	1.7
Lactic acid bacteria	2.6–4.3	2.7
Yeasts and molds	2.5–3.2	2.4

Source: Reprinted with permission from M.J.R. Nout and F.M. Rombouts, Fermentative Preservation of Plant Foods, *Journal of Applied Bacteriology*, Symposium Supplement, Vol. 73, pp. 136S–147S, © 1992, Blackwell Science, Ltd.

26.3.2 Fermentation of Maize To Produce Kenkey

Kenkey is the major staple food of Ghana. It is eaten as a kind of maize bread at almost all mealtimes. Kenkey is produced at household level in small batches (5 to 10 kg), but usually in larger quantities (batches of 100 to 2,000 kg) by cottage enterprises and women's cooperatives.

Method of Production

Figure 26-10 shows the sequence of operations of the traditional process. Whole maize is cleaned, soaked, ground roughly, and made into a dough that is fermented at ambient temperatures (25° to 30°C). No starter is used for this fermentation, but it may be presumed that the use of the same equipment and containers may result in the development of a "house flora" that facilitates the onset of the fermentation. The fermented dough is divided into two equal parts, one of which is mixed with water and cooked to gelatinization. This sticky gel (aflata) is mixed and kneaded with the remaining half; aflata serves as cement and moisturizer in the resulting dumpling. This is shaped, wrapped, and cooked immersed in water. Traditional processes demand much time, physical labor, and fuel energy. Technological interventions, as shown in Figure 26-10, can help to accelerate the process and to achieve a shelf-stable intermediate flour mix, which can be turned into fresh kenkey rather quickly. In this case, the water uptake period is shortened by precracking the kernels, and a starter of lactic acid bacteria is used to speed up the fermentation. As there is much interest in time-reducing and shelf-stable consumer products, a dehydrated mix of fermented dough and pre-cooked and drum-dried aflata has been developed, which can be made into kenkey in relatively short time.

Microbiological and Chemical Changes during Fermentation

Figure 26-11 shows the development of lactic acid bacteria and yeasts in the fermenting dough. Molds and catalase-positive bacteria are present in maize, but disappear during the fermentation. Dominating lactic acid bacteria include *Pediococcus cerevisiae*, *Leuconostoc mesenteroides*, and *Lactobacillus fermentum*. Dominating yeasts are *Candida krusei*, and *Saccharomyces cerevisiae*. Dominating molds (during the initial stages and not regarded as functional flora) are: *Penicillium citrinum*, *Aspergillus flavus*, *A. parasiticus*, *A. wentii*, and *Fusarium subglutinans*.

During the fermentation, several chemical changes take place that are catalyzed either by the microorganisms, or by the endogenous enzymes of maize. In Table 26-9, approximate analysis at key processing stages is presented. During the process, the moisture content increases due to the soaking and cooking. While no significant changes occur in crude protein, ash, fiber, and carbohydrates, there is a marked increase of available lysine. As this essential amino acid is limiting in maize, an increase improves the nutritional value of the product. The bioavailability of nutrients is limited by the antinutritional factors phytate, polyphenols, and lectins, which can form chemical complexes with minerals, proteins (including enzymes of the digestive tract), and sometimes with starch. The reduction of the phytate content (Table 26-9) increases the bioavailability of minerals and proteins. The required phosphatase activities have been demonstrated in lactic acid bacteria and are present also as endogenous activity in several plant tissues. Consequently, phytate degradation is not al-

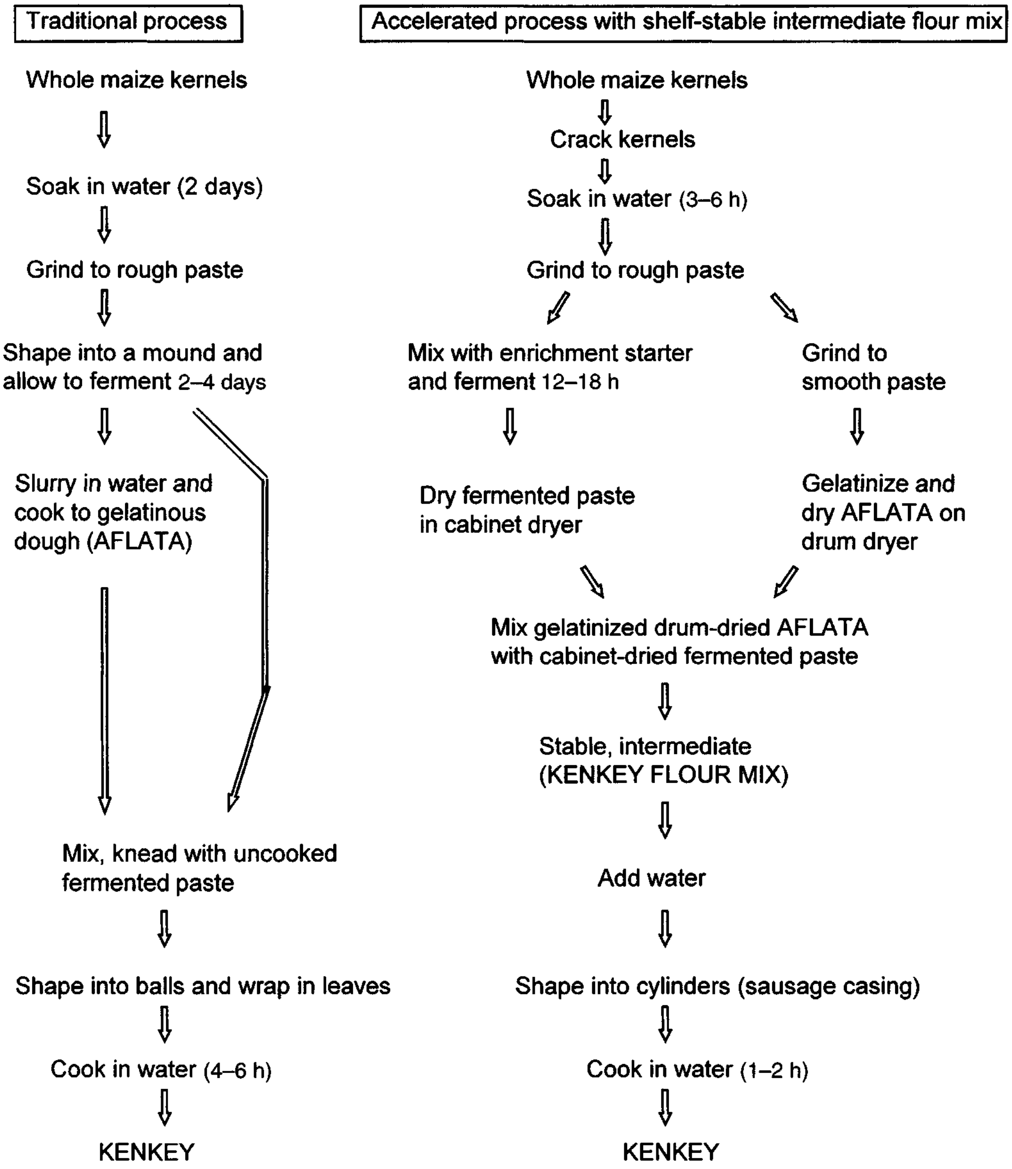
Traditional process
Whole maize kernels
Soak in water (2 days)
Grind to rough paste
Shape into a mound and allow to ferment 2–4 days
Slurry in water and cook to gelatinous dough (AFLATA)
Mix, knead with uncooked fermented paste
Shape into balls and wrap in leaves
Cook in water (4–6 h)
KENKEY
Accelerated process with shelf-stable intermediate flour mix
Whole maize kernels
Crack kernels
Soak in water (3–6 h)
Grind to rough paste
Mix with enrichment starter and ferment 12–18 h
Grind to smooth paste
Dry fermented paste in cabinet dryer
Gelatinize and dry AFLATA on drum dryer
Mix gelatinized drum-dried AFLATA with cabinet-dried fermented paste
Stable, intermediate (KENKEY FLOUR MIX)
Add water
Shape into cylinders (sausage casing)
Cook in water (1–2 h)
KENKEY

Figure 26-10 Kenkey Traditional Process and Innovations. *Source:* Reprinted with permission from P.F. Nche, G.T. Odamtten, M.J.R. Nout, and F.M. Rombouts, Dry Milling and Accelerated Fermentation of Maize for Industrial Production of Kenkey, a Ghanaian Cereal Food, *Journal of Cereal Science,* Vol. 20, pp. 291–298, © 1994, Academic Press Ltd.

ways purely the result of microbial activity. The effect of fermentation on the pasting behavior is also of interest: the texture and cohesion of the cooked product is favored by high peak and set-back viscosities. Important volatile flavor compounds in maize dough fermented with lactobacilli include acetic acid, acetoin, 2,3-butanediol, butanoic acid, lactic acid, 3-methylbutanoic acid, octanoic acid, 2-phenylethanol, and propanoic acid.[50]

Prevention of Spoilage

Traditional kenkey is at risk of mold spoilage during the fermentation of the dough. Superficial mold growth occurs on the dough; this may lead to off-colors, off-taste, and possibly mycotoxin formation. It is important therefore to achieve the lactic acid fermentation as quickly as possible, thereby reducing the available time for the onset of mold growth.

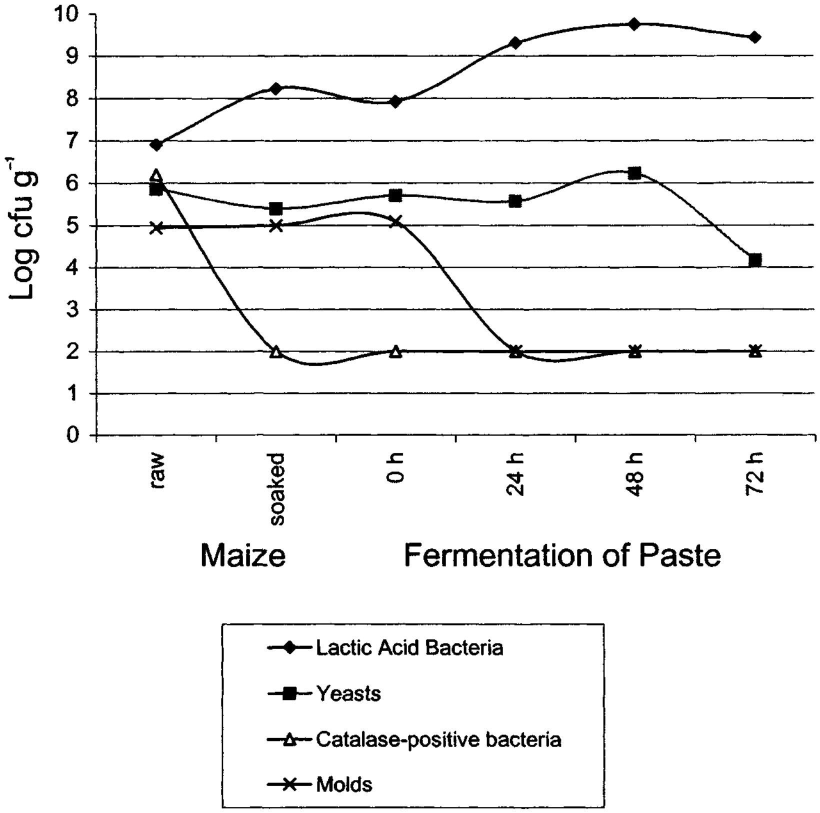

Figure 26-11 Microbiological Changes during the Traditional Kenkey Fermentation Process. *Source:* Data from references 18, 50, 61.

Once wrapped and cooked, kenkey is reasonably well protected against post-processing contamination, and has a shelf life of several days at ambient temperatures, provided the wrapping has not been opened. However, the average consumer will not store kenkey for longer than a day, as staling of the cooked maize gel renders it less palatable.

A long shelf life may be expected from a fermented and dehydrated product (Kenkey-Flour-Mix, Figure 26-10). As a result of dehydration, some losses of volatile organic acid (acetic acid) and esters occur, which might affect the flavor. Drum drying of aflata at 140°C for 0.5 minute reduced the available lysine from 2.36 to 1.59 g 16 g N^{-1}, whereas cabinet drying at 60°C for 3 hours caused no reduction.[88,89] The conclusion is that shelf-stable products can be made, but at the expense of thermal energy, nutritional value, and possibly flavor.

Control of Safety

In general, fermentation improves nutritional properties, makes foods attractive, safeguards food against pathogens, and preserves food. The formation of organic acids and other antimicrobial metabolites, and the competitiveness of actively growing fermentation starters contribute to the destruction or inhibition of bacterial pathogens. Moreover, fermentation usually takes place in the presence of antimicrobial ingredients (spices, salt), or in the context of a sequence of treatments (washing, grinding, cooking, drying). The combined effect of these treatments with fermentation can further increase the safety of food.

In the production of kenkey, the use of maize as raw material always carries the risk of being contaminated with aflatoxins, as well as other mycotoxins, eg, fumonisins. Although some reduction of the mycotoxin levels may be expected from treatments such as soaking and cleaning, lactic acid bacteria have very little impact on the chemically stable molecules of aflatoxins and fumonisins. It is thus essential that the raw material be free of mold growth.

The antimicrobial activity caused by the combined effects of acids, compounds sensitive to pro-

Table 26-9 Chemical Changes during the Kenkey Fermentation Process

	Moisture (% w/w of total)	*Dry Matter (% w/w of total)*	*Crude Protein (Nx6.25) (% w/w of dry matter)*	*Available Lysine (g/16 g N)*	*Crude Fat (% w/w of dry matter)*	*Ash (% w/w of dry matter)*	*Crude Fiber (% w/w of dry matter)*	*Carbohydrates (% w/w of dry matter)*	*Pasting Behavior (Brabender Units)*		*Phytate (% w/w of dry matter)*
									Peak Viscosity	*Set-back Viscosity*	
Raw maize	15.0	85.0	10.7	1.30	4.9	1.5	1.7	81.2	200	500	0.73
Soaked	32.4	67.6	11.4	1.57	4.0	1.5	1.8	81.3			
Fermented dough	51.0	49.0	11.7	2.33	1.5	1.5	1.7	83.6	800	2000	0.31
Kenkey	64.5	35.5	11.6	3.30	1.4	1.5–3.1	1.7	83.2			

Source: Data from references 78, 85, 89, 90.

teolytic enzymes, and other compounds with antimicrobial activity was stronger in mixed culture naturally fermented than in single pure culture fermented kenkey. This means that introduction of pure cultures as starters may impose a risk to the microbiological safety of the product.[113]

As maize contains approximately 10% protein, this could serve as a precursor for the formation of toxic biogenic amines such as histamine. However, there is evidence that levels of biogenic amines in kenkey are well below tolerable levels (about 200 mg kg^{-1} for histamine).[95,105]

In conclusion, kenkey may be regarded as a relatively safe product, providing the raw material was of good quality and the lactic acid fermentation has been rapid. A main reason for the safety of this product is that it is cooked in its packaging material before consumption.

26.3.3 Fermentation of Cereal Flours To Produce Sourdough

Method of Production

Wheat doughs obtain their characteristic elastic and extensible properties from the wheat gluten proteins. The fermentation of wheat dough with added bakers' yeast, *Saccharomyces cerevisiae*, and its subsequent transformation into bread, is discussed in Chapter 28. Despite their lack of elastic gluten, other cereals can also be made into doughs. Rye, for instance, is used as a bread cereal despite its gluten deficiency. As rye doughs tend to form firm gels at reduced pH, the dough and baking properties of rye are enhanced by acid conditions such as prevail in sourdough bread.

Figure 26-12 shows the principle of the manufacture, maintenance, and utilization of sourdough in bread making. Many regional variations occur, but all within the general principles. Sourdough starter cultures are added as pure cultures or spontaneously fermented dough to a fresh flour-water mixture. Depending on temperature, dough yield (water content), presence of salt, and level of inoculation, the fermentation will proceed for 2 to 12 hours at temperatures ranging from 18° to 30°C. Inoculation is often performed with 5% to 10% w/w of previously fermented dough. This practice is referred to as "back-slopping." As a leavening agent in bread making, 10% to 20% w/w of mature (fermented) sourdough is added as dough ingredient. The addition of salt to the bread dough will reduce the speed of fermentation, so usually dough fermentations (carried out in subsequent stages at different temperatures) take a total of 3 to 10 hours. As a result of the activities of lactic acid bacteria and yeasts, gas will be produced as well as organic acids and volatile flavor components. The ratio of lactic:acetic acid is referred to as fermentation quotient (FQ). Higher concentrations of acetic acid result in a sharper sour taste and longer shelf life.

Microbiological and Chemical Changes during Fermentation

Cereal sourdoughs are enrichment cultures of relatively stable combinations of yeasts and (mainly) heterofermentative lactic acid bacteria. Depending on the type and composition of the flour, dough-making conditions, and incubation environment, rather characteristic mutualistic combinations develop during the long periods (months or years) that sourdoughs are maintained. Typical lactic acid bacteria and yeasts encountered are listed in Exhibit 26-1.

Wild strains of sourdough lactobacilli are specialized toward assimilation of maltose, the major assimilable carbon source in cereal dough.[167] A regular supply of maltose is generated by the endogenous cereal amylases. In principle, sugar transport in lactic acid bacteria can occur via (1) high affinity primary transport or phosphoenolpyruvate:sugar phosphotransferase systems both at the expense of ATP and inhibited at pH <6; or (2) low affinity secondary transport systems driven by (electro)-chemical gradients, which are effective at relatively high sugar concentrations. In maltose-grown *L. sanfrancisco* maltose uptake is via maltose-H^+ symport, thus at lower pH the proton-motive force will be increased and so will be the maltose uptake. Within the cell, maltose is phosphorylated and split into glucose-1P and free glucose. About 50% of the latter leaves the cell through a glucose uniport.[87] When all maltose has been taken up, the exported glucose will be assimilated in pure cultures. However, in sourdough the released glucose will be assimilated by yeasts. In sourdough, glucose is exported at the same rate as maltose is imported, reaching glucose levels up to 13 mM.[153]

In the presence of sufficient maltose, maltosephosphorylase, produced by lactobacilli, is not glucose-repressed. This factor is considered essential for the domination of the lactobacilli over microorganisms competing for the same carbon source (ie, maltose). As most other maltose-assimilating organisms are glucose-repressed, the export of glucose would thus serve as an indirect and temporary inhibitor. Nevertheless, maltose-assimilating *S. cerevisiae* competes with *L. brevis* ssp. *lindneri* resulting in re-

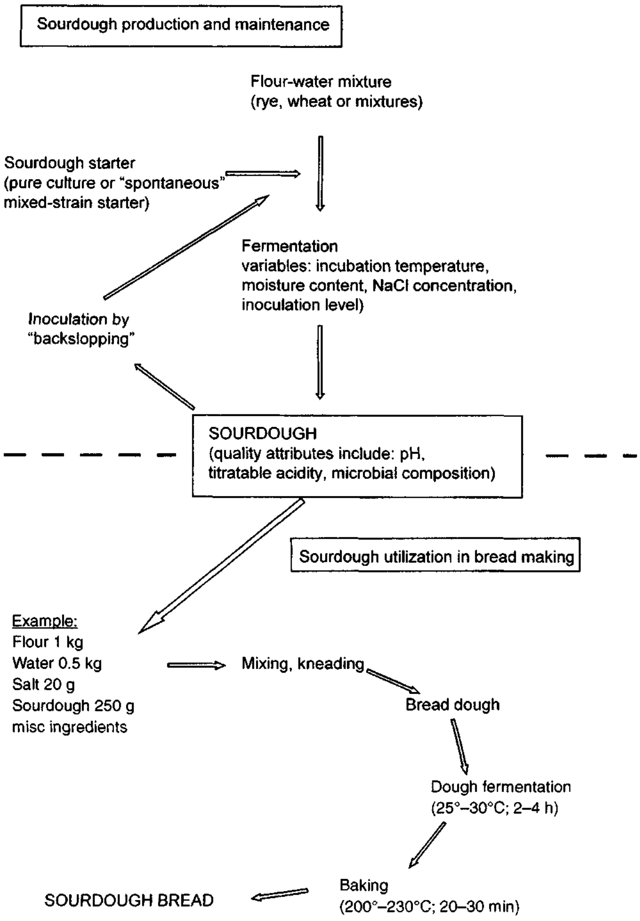
Sourdough production and maintenance
Flour-water mixture
(rye, wheat or mixtures)
Sourdough starter
(pure culture or "spontaneous" mixed-strain starter)
Fermentation
variables: incubation temperature, moisture content, NaCl concentration, inoculation level)
Inoculation by "backslopping"
SOURDOUGH
(quality attributes include: pH, titratable acidity, microbial composition)
Sourdough utilization in bread making
Example:
Flour 1 kg
Water 0.5 kg
Salt 20 g
Sourdough 250 g
misc ingredients
Mixing, kneading
Bread dough
Dough fermentation
(25°–30°C; 2–4 h)
Baking
(200°–230°C; 20–30 min)
SOURDOUGH BREAD

Figure 26-12 Sourdough Fermentation and Utilization. *Source:* Adapted with permission from G. Spicher and H. Stephan, *Sourdough Handbook, Biology, Biochemistry, Technology,* 2nd ed., © 1984, BBV Wirtschaftsinformationen GmbH.

duced bacterial growth and organic acid production.[49] It was also suggested that lactic acid bacteria may produce antibiotics and thus support the stability of the sourdough population, but no direct evidence has been presented to confirm or contradict this hypothesis. Most yeasts present in sourdough are quite robust. They tolerate the concentration of acids present, but they do not appear to compete with the lactic acid bacteria because most of the prevailing species cannot assimilate maltose.

Yeasts may have a role as suppliers of growth factors; *S. cerevisiae* and *S. exiguus* excrete alanine, glutamic acid, leucine, and valine during the stationary phase of growth, and these amino acids stimulated growth of *L. brevis* ssp. *lindneri.* Lactic acid bacteria from sourdoughs require vitamins and varying numbers of amino acids, which could be supplied either by soluble matter from fresh yeast, a peptide, or live yeast cells.[94] It was also observed that in wheat doughs, proline and arginine are assimilated faster by mixed yeast–lactic acid bacteria cultures than by the individual microbial components. However, considerable levels of free amino acids are generated in dough by proteolytic enzymes of the cereal grain, and yeasts (*S. cerevisiae*) consume, rather than release, amino acids.[19] It appears that the extent of acidification of lactic fermentations of cereal doughs will, *inter alia,* depend on the availability of micronutrients for lactic acid bacteria. If the latter are deficient in the raw material to be fermented, they will have to be supplied by yeast cells. Consequently, an equilibrium situation will be established with final pH values and acetic acid concentrations enabling adequate yeast growth. It remains still to be clarified to what extent yeasts play a role as growth stimulators for sourdough lactic acid bacteria.[97]

Table 26-10 shows data illustrating the kinetics of acidification. At higher fermentation temperatures, the fermentation quotient (FQ) is usually higher (up to 5–7).

Prevention of Spoilage

The shelf life of bread is affected mainly by two microbiological aspects, namely mold growth and ropiness. Mold growth by *Rhizopus nigricans, Neurospora sitophila, Aspergillus,* and *Penicillium* spp. is most common on bread. The presence of organic acids, mainly acetic acid, that are undissociated at the prevailing pH (3.5–4.5), will delay the onset of mold spoilage in sourdough bread. Also, wrapping

Exhibit 26-1 Frequently Encountered Microorganisms in Sourdoughs

Lactic Acid Bacteria 10^9–10^{10} cfu g^{-1}	**Yeasts** 10^6–10^8 cfu g^{-1}
Lactobacillus acidophilus	*Candida holmii*
L. alimentarius	*C. krusei*
L. brevis	*C. milleri*
L. büchneri	*Pichia saitoi*
L. casei	*Saccharomyces cerevisiae*
L. delbrückii	
L. farciminis	*S. exiguus*
L. fermentum	
L. fructivorans	
L. plantarum	
L. sanfrancisco (brevis var. *lindneri)*	

bread in water vapor–permeable kraft paper, rather than in plastic bags, delays mold growth.

Ropiness in regular yeast-leavened bread is a type of spoilage that is caused by survival and growth of *Bacillus subtilis* and *B. mesentericus*, which produce capsular materials and degrade bread protein and starch, resulting in a sticky, threadpulling crumb with a typical pineapple smell. In sourdough bread, this type of spoilage occurs only very seldom because the sensitivity to heat of *Bacillus* spores is significantly increased at the pH prevailing in sourdough, compared with that in pH-neutral doughs. The maximum crumb temperature (usually occurring during 15 to 30 minutes) during baking is approximately 97°C, and decimal reduction times of *B. subtilis* spores at 97°C were determined in sourdough (Table 26-11). Sourdough was made with a representative inoculation level of 10% mature sourdough as a starter. After fermentation and baking, the resulting bread was incubated for 1 week at 37°C.

The data show that regular sourdough bread has an adequate antimicrobial effect to prevent ropiness. At the low level of contamination, the ratio lactic:acetic acid was not critical; at the higher level of *B. subtilis* only the lower FQ (signifying more acetic acid) was effective.

26.3.4 Fermentation of Cassava To Produce Gari and Similar Products

Method of Production

Cassava is highly perishable. In addition, bitter cassava varieties contain potentially toxic substances. Processing of cassava therefore has two major objectives: prolongation of shelf life by dehydration, and detoxification by degradation of the toxic principles prior to dehydration. Additional objectives include the formation of desirable smell and taste. Figure 26-13 illustrates the major steps involved in the production of gari, lafun, and mold-fermented cassava flour.

Cassava processing for detoxification involves some degree of size reduction combined with submerged fermentation. After peeling, the roots are cut into pieces and stored under water for several days, during which a retting process takes place. The softened cassava pieces are taken out of the water, sun dried, and subsequently milled to flour (eg, Nigerian "lafun"), or used to prepare creamy moist pastes such as "fu-fu."

Alternatively, for gari production, washed and peeled cassava can be grated to pulp and left to ferment in jute bags under pressure to exclude air. All fermentations take place at ambient temperatures (25°–35°C), and are carried out on a household or cottage scale in batches of several hundreds of kilos. Gari is also made on a semi-industrial scale producing several tons daily. On all scales of processing starters are used. These usually consist of fermented material of a previous batch. After fermentation of the pulp, it can be steamed for direct consumption (Ivorian "attiéké") or dry roasted to yield a dry pregelatinized instant breakfast meal (Nigerian "gari").

In several African countries, a solid-substrate fermentation process is carried out at the farm level. Cassava roots are washed, peeled, and cut into large pieces. These are made into a big heap, which is kept covered for a few days with a plastic sheet or suitable leaf material. When sufficient mold growth has occurred (criteria used are color, caused by mold sporulation, and odor) the cover is removed, the pieces are

Table 26-10 Acidification in a Commercial Multistrain Sourdough ("Reinzuchtsauer") Fermenting at 25°C

Fermentation (time (h))	*pH*	*TTA (Total Titratable Acid) (as lactic acid (%w/w))*	*LA (Lactic Acid) (mmol kg^{-1})*	*AA (Acetic Acid) (mmol kg^{-1})*	*FQ (Fermentation Quotient) (Mol. LA/AA)*
0	5.5	0.27	11	11	1.0
3	5.1	0.45	22	22.4	0.98
6	4.3	0.72	44	28	1.57
9	3.9	0.99	66	30	2.2
12	3.75	1.17	71	33	2.2
15	3.7	1.26	74	41.6	1.78
18	3.6	1.44	79	50	1.58
21	3.6	1.53	83	58	1.43
24	3.6	1.62	89	66.6	1.35

Source: Recalculated from data of G. Spicher and H. Stephan, *Sourdough Handbook, Biology, Biochemistry, Technology*, 2nd ed., BBV Wirtschaftsinformationen GmbH.

Table 26-11 Effect of Sourdough Addition on Heat Sensitivity of *Bacillus subtilis* in Bread Dough and on Rope Formation

Sourdough Level in Bread Dough (%)	Bacillus subtillis *Contamination (spores g^{-1} dough)*	*TTA (% w/w)*	*pH*	*FQ*	*D_{97} (min.)*	*Ropiness*
0	10	2.3	5.8	ND	143.0	+
10	10	4.1	4.9	3.5	15.9	–
10	10	ND	4.7	1.0	ND	–
10	500	ND	4.7	3.5	ND	+
10	500	ND	4.7	1.0	ND	–

TTA = Total titratable acidity, expressed as lactic acid.
FQ = Fermentation quotient (mol. lactate:acetate).
D_{97} = Decimal reduction time at 97°C.
Ropiness = After incubation at 37°C for 7 days.
ND = Not determined.

Source: Reprinted with permission from W. Röcken and P.A. Voysey, Sour-Dough Fermentation in Bread Making, *Journal of Applied Bacteriology*, Supplement, Vol. 79, pp. S38–S48, © 1995, Blackwell Science, Ltd.

scraped to remove excessive mold, and spread out in the open to dry. Finally, the pieces are ground to a flour, which is used to prepare cooked porridges.

Microbiological and Chemical Changes during Fermentation

The submerged fermentation of cassava pieces in water during the preparation of lafun is dominated first by homofermentative lactic acid bacteria (eg, *Lactobacillus plantarum*) followed by heterofermenters (*L. brevis*). *Clostridium* spp. usually occur in retted cassava; they are responsible for the typical butyric acid flavor,[12] and possibly also for the softening of the cassava tissue.

During fermentation of cassava pulp for gari production lactic acid bacteria (including *L. plantarum* and *L. brevis*) dominate during the fermentation, but other bacteria (*Bacillus*, *Staphylococcus* spp.) and yeasts have been found also in fermented pulp (Table 26-12).

As a result of lactic acid fermentation, the pH decreases and acid is formed at the expense of reducing sugars (Table 26-13). The flavor added by the fermentation adds to the popular taste of the product. Gari consists mainly of carbohydrates (starch) (Table 26-13). Swelling capacity is an important quality characteristic of gari. Having been roasted, its starch component has gelatinized and the product will absorb water readily, like an instant breakfast cereal. The higher the swelling capacity, the better the palatability of prepared gari.

During the sweating period in the production of mold-fermented cassava flour, moisture migrates from the center of the cassava pieces to the surface, but the cassava will retain sufficient water to enable a profuse microbial growth, consisting of rapid-growing molds (*Rhizopus*, *Neurospora* spp.) and bacteria (eg, *Bacillus* spp.). Cell wall degradation occurs as a result of fungal and bacterial enzyme activity, resulting in considerable softening.

Prevention of Spoilage

The shelf life of gari depends mainly on its water activity. Good quality gari has a water activity of 0.5–0.7 and a shelf life of 3 to 6 months. Table 26-14 lists a number of molds frequently encountered in gari. When added as a pure culture to gari that had been previously moistened, the fast-growing *Rhizopus* caused deterioration in 2 days; other slower-growing molds (*A. niger*, *A. candidus*, *A. tamarii*, *A. versicolor*, *A. flavus*, and *Cladosporium* spp.) needed 3 days. Adequate packaging is also essential to keep the water activity of the dry product low. In practice, industrially produced gari is bagged in woven polypropylene bags for distribution, and in polythene bags for retail purposes. Preferred traditional storage in the household is in recycled hermetically sealed tins.

Control of Safety

Consumption of raw, or inadequately processed, bitter cassava varieties may lead to ingestion of prussic acid (HCN). Mastication of raw cassava tissue containing linamarin and/or lotaustralin initiates the endogenous enzymic degradation of the glycoside, releasing unstable cyanohydrin that splits into acetone and HCN. Incorrectly processed cassava may contain residual levels of HCN. The toxic effects of HCN can be chronic at low doses, leading to paralysis and iodine deficiencies with concomitant symptoms of cretinism and mental retardation. Acute HCN intoxication may lead to respiratory failure and death. Thus, the main safety issue with

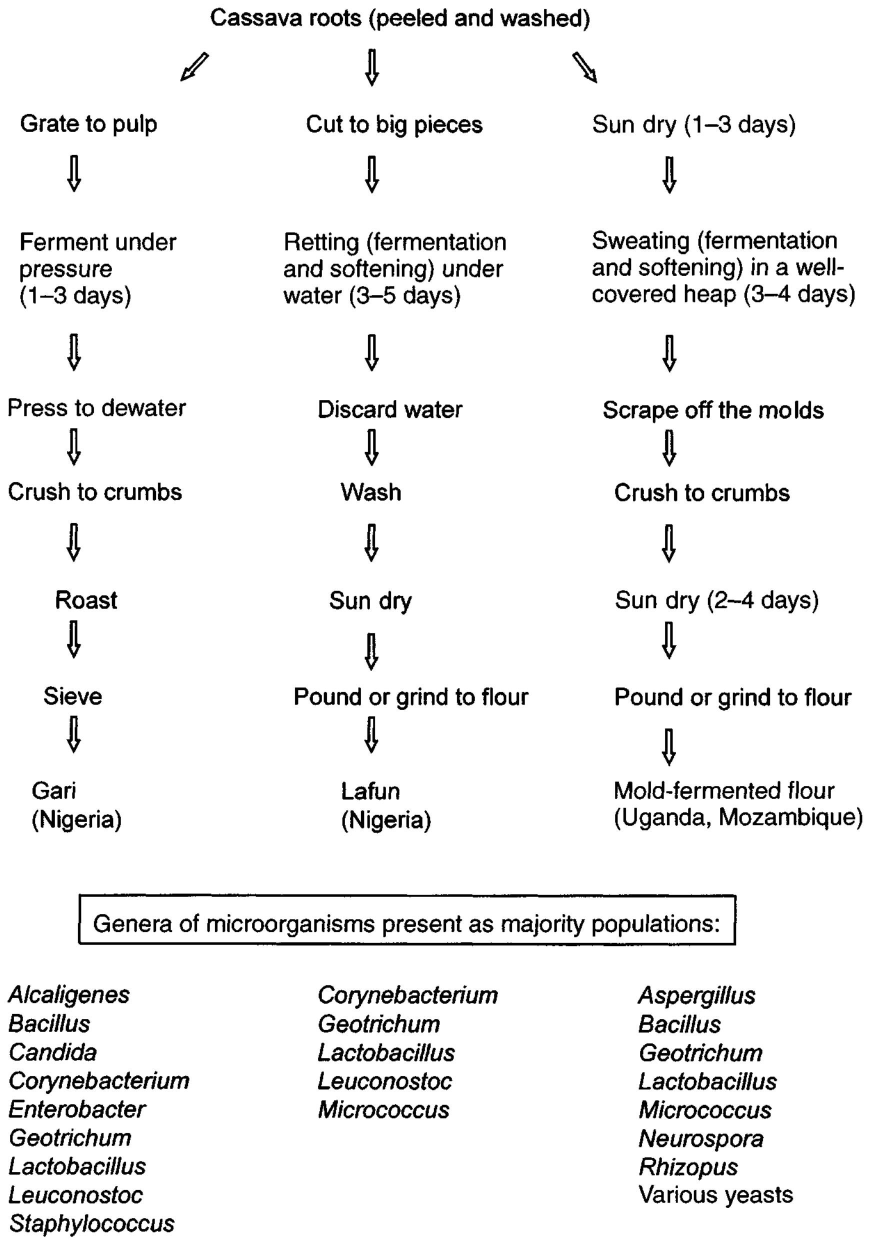

Figure 26-13 Prodution of Fermented Cassava Products and Major Microorganisms Involved. *Source:* Data from references 3,11,32–34,115,164.

cassava products is whether adequate detoxification of the cyanogenic glycosides has been achieved.

Within the cassava root, the cyanogenic glycosides are distributed unevenly, but they are present in all parts of the root, and are located in the cytoplasm. The endogenous enzymes able to degrade them (glycosidases, linamarinases) are cell-membrane bound, but come into contact with the glycosides upon loss of integrity of the cell due to mechanical damage (cutting, grinding) or enzymic degradation (physiological reactions leading to wilting, or microbial retting).

Although cassava can be fermented in different ways, experimental evidence shows that in gari, cyanide removal is a consequence of the activity of naturally occurring plant enzymes and process operations rather than microbial activity. Although about 50% of the lactic acid bacteria in fermenting cassava pulp are able to degrade linamarin, the microbial effects on linamarin are negligible during

Table 26-12 Microbiological Changes during the Manufacturing Stages of Gari from Cassava

Stage	*Count of Mesophilic Bacteria (Log cfu g^{-1})*	*Lactic Acid Bacteria (Log cfu g^{-1})*	*Groups of Microorganisms Encountered*						
			1	*2*	*3*	*4*	*5*	*6*	*7*
Grated to pulp	6.0	6.0	++	+	++			++	+++
Fermentation at ambient temperature (25°–30°C):									
For 1day	6.7	8.8	++					+	++
For 2 days	5.9	8.7	++					++	++
For 3 days	5.8	8.7	+			+	+	+++	
After dewatering	5.9	9.0	++			++		++	++

1: *Bacillus* spp.; 2: Enterobacteriaceae; 3: *Acinetobacter;* 4: *Staphylococcus*; 5: yeasts; 6: *Lactobacillus plantarum*; 7: *Lactobacillus brevis*;
+++ : strongly dominating; ++: dominating; +: present.

Source: Reprinted with permission from A. Westby and D.R. Twiddy, Characterization of Gari and Fu-Fu Preparation Procedures in Nigeria, *World Journal of Microbiology and Biotechnology*, Vol. 8, pp. 175–182, © 1992, Rapid Communications in Oxford.

this type of fermentation, and acidification is inhibitory to the degradation of linamarin by endogenous plant enzymes. In other cassava products, such as lafun and fungal fermented flour, molds soften the plant tissues, thus facilitating the reduction in cyanogen content.

Table 26-15 summarizes data on levels, toxicity, and Codex Alimentarius recommended standards for cyanide in gari, and the effect of gari processing on cyanogenic substances in sweet and bitter varieties. In both types of cassava, detoxification is achieved after grating. Industrial-scale processing with finer grating and better squeezing of the plant juice (by screw press) results in lower residual cyanide levels than traditional production. In both cases, bound cyanide has been more or less detoxified; residues consist of free HCN and are in line with the recommendation.

26.4 LEGUMES, OILSEEDS, AND TREEBORNE SEEDS (PROTEINACEOUS PLANT FOODS)

26.4.1 General Aspects

Range of Plant Materials Used and Products Produced

Leguminous seeds, oilseeds, and, to some extent, treeborne seeds are used as a source of dietary protein.

Table 26-13 Chemical Changes during Fermentation, and Composition of Gari Made of Cassava

	pH	*TTA, Expressed As Lactic Acid (% w/w of total weight)*	*Reducing Sugars (% w/w of total weight)*
During fermentation:			
Grated pulp	6.2	0.27	0.31
1 day fermented	5.5	0.36	0.62
2 days	4.5	0.49	0.44
3 days	4.0	0.76	0.28
Composition of gari:			
Dry matter (% w/w of total)	85.6–91.8		
Crude fat (% w/w)	0.1		
Crude protein (% w/w)	0.9–1.5		
Crude fiber (% w/w)	1.4–2.3		
Ash (% w/w)	0.9–1.4		
Carbohydrate (% w/w by difference)	81.8–87.0		
Swelling capacity (%)	300–320		

Source: Data from O.O. Onyekwere et al., Industrialization of Gari Fermentation, in *Industrialization of Indigenous Fermented Foods*, K.H. Steinkraus, ed., pp. 363–410, © 1989, Marcel Dekker, Inc.

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Table 26-14 The Occurrence of Molds on Market Gari, and the Ability of the Molds When Inoculated at Low Levels To Cause Spoilage of Gari Moistened to 15% and Incubated at 30°C

	Occurrence	*Days To Cause Deterioration*
Penicillium variabele	++[a]	5
Rhizopus nigricans	++	2
Aspergillus niger	++	3
A. candidus	+[b]	3
A. tamarii	+	3
A. versicolor	+	3
A. flavus	+	3
Fusarium spp.	±[c]	No effect
Curvularia spp.	±	5
Cladosporium spp.	±	3

[a] ++ = Log cfu g^{-1} approx. 2–3.

[b] + = Log cfu g^{-1} approx. 2.

[c] ± = Log cfu g^{-1} approx 1.

Source: Data from O.O. Onyekwere et al., Industrialization of Gari Fermentation, in *Industrialization of Indigenous Fermented Foods*, K.H. Steinkraus, ed., pp. 363–410, © 1989, Marcel Dekker, Inc.

Especially when animal proteins are less available or too expensive, plant proteins may serve as meat substitutes. Table 26-16 provides an overview of proteinaceous plant materials of which fermented foods are made. These proteinaceous plant foods have a protein content of 30% to 50% (w/w) (Table 26-17).

As a dietary source of protein, the most important leguminous seeds include cowpea (*Vigna unguiculata*), gram (*Phaseolus* spp.), and pea (*Pisum* spp.). Black and green gram are widely used in Asia, while cowpea is more popular in Africa and the Caribbean. An important source of dietary protein and fat are the various oilseeds of which soya beans (*Glycine max*) are the most important. Whereas soya beans are known and used worldwide, their traditional fermentation processes originate in Southeast Asia. Several types of groundnut (*Arachis hypogaea*) and African oilbean (*Pentaclethra macrophylla*) are used in Asian and African fermented foods. In addition, sunflower seed (*Helianthus* spp.) and agricultural by-products, eg, oilseed presscakes are traditionally converted by fermentation into food materials. The seeds of some trees (*Parkia biglobosa* or locust bean) are gathered and used in traditional fermentations in West Africa.

Range of Types of Fermentation

Three categories of fermented commodities can be distinguished. (1) Products in which extensive hydrolysis and degradation of protein has occurred result in a liquid hydrolysate. These include shoyu and kecap (soya sauces) and are characterized by their high content of soluble nitrogen compounds, particularly free amino acids, which act as flavor enhancers and thus are used as flavorings or condiments. (2) Products in which the integrity of the beans has remained intact have undergone extensive enzymatic modification. The digestibility of their protein has been greatly improved by processing, and a moderate extent of proteolysis contributes to a meat-like flavor. The fermentation of these typical meat-substituting side dishes can be achieved with molds (examples of products are oncom, taoco, miso, tempe) or with bacilli (products including ugba, kinema, itohiki-natto, soumbala, dawadawa, and netetou). (3) Protein-enriched fermented starchy materials, eg, fermentation of rice flour and pea or bean flour, produce Indian idli. The addition of proteinaceous seeds to cereals increases the total protein content, and improves the pattern of amino acids because the lysine deficiency of cereal proteins is compensated by lysine of the legume protein. The protein-enriched cereals undergo a lactic acid fermentation or mixed lactic-alcoholic fermentation. This may entail considerably less proteolysis than in the other two product groups.

Status

Industrialization of fermentation processes is most advanced with Japanese shoyu. In Japan only, the 1989 production was estimated[45] at about 1 to 2 million tons. Soya sauce is also manufactured on an industrial scale in the United States and in Europe. Of the products in the second category, miso is probably produced on the largest industrial scale. Production of industrial and homemade miso in Japan in 1985 was of the order of 600,000 and 60,000 tons, respectively.[29]

Tempe is also consumed in considerable quantities mainly in Indonesia; estimates range from 150,000 to 500,000 tons yearly.[98] Some tempe is produced on small-industrial scale (about 1 ton daily); most tempe, and other products in the second category, are produced on a household scale (10 to 200 kg daily). Category 3 products such as idli are staple foods in India, for example. Industrialization has resulted in the production of packaged, shelf-stable, labor-saving intermediate products such as "instant idli mix" consisting of dried ingredients. Fermentation and finishing of the product still needs to be done in the household.

Composition of Starting Material

Table 26-17 presents overall ranges of approximate composition of leguminous seeds and oilseeds.

Table 26-15 Cyanide in Cassava and Gari

Cassava tubers: *15–500 mg HCN equivalent kg^{-1} fresh weight*
Toxicity: *50–100 mg dose lethal to human adult*
Codex Alimentarius tolerable level in food: *10 mg CN equivalent kg^{-1} dry weight*

	HCN Equivalent mg kg^{-1} Dry Weight		
	Total CN	*Free HCN*	*Bound Cyanide*[a]
Bitter cassava variety			
Grated pulp	1375	1144	231
Gari (24 h; screw press)[b]	4.5	3.8	0.6
Gari (72 h; traditional)[c]	12.3	12.5	0
Sweet cassava variety			
Grated pulp	169	155	15
Gari (24 h; screw press)	3.4	3.0	0.3
Gari (72 h; traditional)	11.5	12.0	0

[a] Total CN = cyanide bound as the glycosides linamarin and lotaustralin and as cyanohydrins, plus free HCN.

[b] Fermented for 24 h, followed by dewatering using industrial screw press.

[c] Fermented for 72 h, during which dewatering takes place under the weight of stones.

Source: Data from G. Padmaja, Cyanide Detoxification in Cassava for Food and Feed Uses, *Critical Reviews in Food Science and Nutrition*, Vol. 35, pp. 299–399, © 1995.

The digestibility of plant protein is often adversely affected by ANFs, which occur naturally in seeds. Processing and fermentation, in particular, can increase the digestibility of proteinaceous seeds.

The epiphytic microflora of dry seeds does not differ significantly from that of dry cereal grains. Predominant microorganisms include several groups of strictly aerobic bacteria and their spores, lactic acid bacteria, some yeasts, and fungal spores.

26.4.2 Fungal Fermentation To Produce Tempe

Method of Production

Tempe is principally a fungal solid-substrate fermented type of food. However, the microbial composition of tempe also includes high numbers of bacteria and yeasts; some of these may influence acceptability and product safety, as will be discussed in the next sections. Although most tempe is made from yellow-seeded soya beans (the product is then called "tempe kedele"), tempe can also be made from various leguminous beans, seeds, cereals, and presscakes (residues of oilpressing from groundnuts, coconut, or other lipid-rich materials). The latter category especially provides a nutritious, yet affordable, food to low-income groups of the population.

To produce tempe, the raw materials are soaked, dehulled, cooked, and then mixed with a starter of sporangiospores, mainly of *Rhizopus oligosporus* (Figure 26-14). The inoculated beans are packed, or wrapped in leaves or plastic sheets with minuscule (approximately 1 mm diameter) perforations. The sporangiospores germinate, and produce profuse mycelium growth, but no sporulation, throughout the substrate, thus "knitting" it together to form a cohesive, sliceable cake. The extent of perforation, the shape and size of the package, and the porosity of the granular product limit the heat and mass transfer to and from the center of the package.

As the actively growing mycelium requires atmospheric oxygen and releases carbon dioxide and heat, these heat and mass transfer limitations affect the availability of oxygen required for growth, the accumulation of CO_2, and the increase in temperature. Traditional manufacturers achieve the desired quality by, for example, adjusting thickness of fermenting beds of raw materials, and reducing the access of air to the material according to the ambient environmental conditions.

Prior to consumption, tempe is heated, eg, in stews, or fried to highly popular "tempe kripik" crunchy chips. Dehydration and grinding yields tempe powder, which can be used to enrich low-protein foods.

The starter used ("usar"; Figure 26-14) for the manufacture of traditional tempe is an interesting example of simple household-scale technology. A rough-surfaced natural carrier is used on which the mold mycelium is grown. Although various plant leaves and even kraft paper can be used, the most popular leaf is that of the "waru" tree (*Hibiscus tileaceus* var. *tileaceus*), which carries microscopically small hairs at the underside, and which does not become too brittle when dried. Some cooked soya beans are inoculated with previous starter, and are sandwiched between two waru leaves (undersides turned inward). When the sandwiches have been incubated for 2 days under humid, warm (30°C) conditions, good mycelial growth and heavy sporulation are visible. At that stage, the sandwiches are opened and kept for a few days exposed in order to sun dry. Packages of usar (the dried leaves with inoculum) are sold in local markets and viability of spores is retained for several months at ambient temperature. The normal epiphytic fungi on waru leaves include typical soil fungi such as *Cladosporium* spp. In Indonesia, the leaves are also heavily loaded with *Rhizopus* and *Mucor* spp. probably because of air dispersion caused by widespread use of fungal fermentations. In the tempe fermentation,

Table 26-16 Fermented Foods Made from Proteinaceous Food Plants

Raw Material	Name and Origin of Fermented Product	Principle of Manufacturing Process	Characteristics and Use of Product	Predominant Microorganisms	References
Category (1): Liquid hydrolysates					
Soya bean (*Glycine max*)	Shoyu (Orient); kecap (Indonesia) soya sauce	Mix autoclaved soya beans and roasted wheat, inoculate with koji starter, solid-state fermentation 2–3 days, followed by brine fermentation (several months), aging, pressing, pasteurization (in Indonesia, no wheat is used).	Liquid hydrolysate; condiment	*Aspergillus oryzae, Tetragenococcus halophila, Zygosaccharomyces rouxii*	1, 75, 126–130
Category (2): Meat-substituting side dishes with a firm consistency of the beans					
African oilbean (*Pentaclethra macrophylla*)	Ugba (West Africa)	Boil seeds 4–5 h, extract and cook cotyledons overnight, cool, wash, soak 6 h, slice kernels, add salt, wrap in leaves, ferment about 5 days.	Condiment	*Bacillus, Staphylococcus, Micrococcus, Leuconostoc* spp.	91, 111
Bambara groundnut (*Voandzeia subterranea*)	Dawadawa-like products	Soak 24 h, dehull, boil cotyledons 15 min., drain, ferment 4 days.	Condiment	*B. subtilis, B. licheniformis*	8
Groundnut (*Arachis hypogaea*)	Oncom (Indonesia)	Soak groundnut presscake, drain, knead with cassava or soya bean residue, steam the dough, press in mold to brick shape, inoculate with dried previous oncom.	Meat substitute	*Neurospora intermedia, N. sitophila*	71, 96
Locust bean (*Parkia biglobosa*)	Soumbala (Mali, Burkina Faso) Dawadawa/Daddawa/Iru (Nigeria) Netetou (Senegal)	Boil beans 12 h, dehull, boil cotyledons again 2 h, drain, ferment 36 h, pound to paste.	Condiment for soups and stews	*Bacillus licheniformis, B. stearothermophilus, B. brevis, B. mycoides, B. subtilis*	5, 107–110
Soya bean (*G. max*)	Taoco (Indonesia) Hama-natto (Japan)	Soak, dehull, boil soya beans, inoculate with tempe starter, ferment 2–5 days, sun dry, ferment in brine 3–4 weeks, cook.	Flavoring agent	*Aspergillus oryzae, Hansenula* spp., *Lactobacillus delbrückii, Rhizopus oligosporus, R. oryzae, Zygosaccharomyces soyae*	74, 81, 152, 162
Soya bean (*G. max*)	Miso (Japan)	Mix crushed, steamed soya beans with salt and mold-fermented rice or barley to paste. Ferment.	Pasty meat extender and seasoning	*Aspergillus oryzae, Saccharomyces rouxii, Tetragenococcus halophila*	46, 152
Soya bean (*G. max*)	Kinema (India, Nepal)	Soak soya beans, cook, crush, dehull, wrap in leaves, ferment 1–3 days.	Condiment; side dish	*B. subtilis, Enterococcus faecium, Candida parapsilosis, Geotrichum candidum*	139–142

continues

Table 26-16 Continued

Raw Material	*Name and Origin of Fermented Product*	*Principle of Manufacturing Process*	*Characteristics and Use of Product*	*Predominant Microorganisms*	*References*
Soya bean (*Glycine max*)	Itohiki-Natto (Japan)	Soak, cook soya beans, inoculate, pack, ferment 14–18 h.	Nutritious and viscous protein food	*Bacillus natto*	2, 46, 112, 162
Soya bean (*Glycine max*) Kidney bean (*Phaseolus vulgaris*) Sunflower seed (*Helianthus annuus*)	Tempe (Indonesia)	Soak, dehull, cook soya beans. Inoculate with tempe starter, pack in leaves or perforated sheet, ferment 1–2 days.	Firm cake; meat substitute	*Rhizopus oligosporus*, *Lactobacillus plantarum*, *Klebsiella pneumoniae*, *Saccharomyces dairensis*, *Trichosporon beigelii*	56, 72, 98, 124, 157
Category (3): Protein-enriched fermented starchy materials					
Bengal gram or Chick pea (*Cicer arietinum*) Black gram (*Phaseolus mungo*) Rice flour	Idli (India)	Soak rice flour and gram dhal (flour) separately, then join them and allow natural fermentation; prepare steamed cakes.	Steamed cakes as staple food	*Leuconostoc mesenteroides*, *Streptococcus faecalis*, *Pediococcus cerevisiae*	65, 145, 152

Table 26-17 Chemical Composition and Main Components of the Microbial Flora of Some Proteinaceous Food Plants Used in Fermentation Processes

(per 100 g edible portion)	*Legumes*	*Oilseeds*
Moisture (g)	12	3–6
Crude protein (g)	20–35	15–30
Crude fat (g)	1–2	35–65
Carbohydrates (g)	40–60	10–35
Reducing sugars (g)	2–4	5–10
Calcium (mg)	30–300	20–500
Phosphorus (mg)	400	90–600
Iron (mg)	5–10	1–10
Sodium (mg)	2–10	2–25
Potassium (mg)	1000–1250	400–900
Manganese (mg)	ND[a]	500–800
Vitamin A (I.U.)	0–100	0–100
Thiamine (mg)	0.1–0.9	0.03–1
Riboflavin (mg)	0.1–0.4	0.01–0.3
Niacin (mg)	0.25–0.35	0.6–7
Ascorbic acid (mg)	ND	<10

Epiphytic microflora of legumes and oilseeds ($\log_{10} N\ g^{-1}$):

Bacteria:	
Aerobes	5–7.7
Enterobacteriaceae	3.5
Lactic acid bacteria	4.2
Yeasts and molds	2.5

[a]ND = Not determined.

Source: Reprinted with permission from M.J.R. Nout and F.M. Rombouts, Fermentative Preservation of Plant Foods, *Journal of Applied Bacteriology*, Symposium Supplement, Vol. 73, pp. 136S–147S, © 1992, Blackwell Science, Ltd.

Rhizopus and *Mucor* spp. compete successfully with soil fungi because of their faster growth.[104]

In addition to the traditional use of usar, selected pure cultures of *Rhizopus oligosporus* are grown and diluted into soya flour to give defined starter material for tempe fermentations.[154] Whereas small-scale producers prefer usar because of easy availability and handling, industrialization of tempe manufacture will require the development of better-defined starters.

Microbiological and Chemical Changes during Fermentation

As indicated in the previous section, bacteria and yeasts multiply in tempe during the manufacturing process, and lactic acid bacteria especially play an important role during the first phases of the process. During soaking of the soya beans, fermentable sugars leach into the soak water enabling the growth of acid-producing Gram-positive bacteria (Figure 26-15), including *Lactobacillus casei*, *Staphylococcus epidermidis*, *Enterococcus faecium*, and *Streptococcus dysgalactiae*.

Depending on the soaking conditions, lactic acid bacteria, especially the acid-tolerant species such as *Lactobacillus plantarum*, will dominate the microflora of the soak water either immediately (soak water inoculated) or at the end of the overnight soaking period (succession of naturally occurring microorganisms in the absence of inoculum); Gram-negative bacteria, eg, *Klebsiella pneumoniae*, *Citrobacter diversus*, *Pantoea agglomerans*, *Klebsiella ozaenae*, and *Enterobacter cloacae* are also present in soak water and in tempe. Yeasts, eg, *Pichia burtonii*, *Candida diddensiae*, and *Rhodotorula rubra* were also found in soak water.[86]

Some bacterial acidification of the soak water is desirable because it limits bacterial spoilage during the fungal fermentation stage. Addition of previous soak water as an inoculum results in enrichment of lactic acid bacteria, causes a further decrease in the pH of soya bean soak water (to about 4.5 or 4.0), and lowers the pH of the beans after cooking; potential spoilage and/or food poisoning bacteria are also inhibited. Addition of a pure culture of *Lactobacillus plantarum* at the start of the soaking stage (10^3 cfu ml^{-1} soak water) resulted in tempe with significantly lower counts of Enterobacteriaceae and *Bacillus* spp. ($\log_{10}$ cfu ml^{-1} <1.7) than in tempe obtained from beans that had been soaked without inoculation ($\log_{10}$ cfu ml^{-1} 8.13 and 8.07, respectively).[101]

Tempe making was introduced in the Netherlands by Indonesian immigrants, and the product is manufactured commercially by several small- and medium-scale enterprises. Tempe contains large numbers of bacteria, usually dominated by lactic acid bacteria, but less desirable bacteria are sometimes present in significant numbers (Table 26-18). For instance, in 13% of the samples tested the number of *S. aureus* exceeded 10^5 cfu g^{-1}. *Rhizopus oryzae*, *R. oligosporus*, and *Mucor indicus* are the most important molds. Yeasts may be present at levels of 10^5–10^7 cfu g^{-1}; *Trichosporon* and *Candida* spp. are most frequently encountered.

Fermentation of cooked soya beans to produce tempe (Table 26-19) results in reduction of crude fat, carbohydrates, insoluble fiber, and phytic acid, and in an increase in the levels of a number of vitamins. Additional changes affect the digestibility and nutritive value of the product. During the fermentation, protein nitrogen is enzymatically degraded to small peptides, free amino acids, and amines.[9,55] Soya bean isoflavones (daidzein and glycitein) are hydroxylated[69] or transformed further into 3-hydroxy-

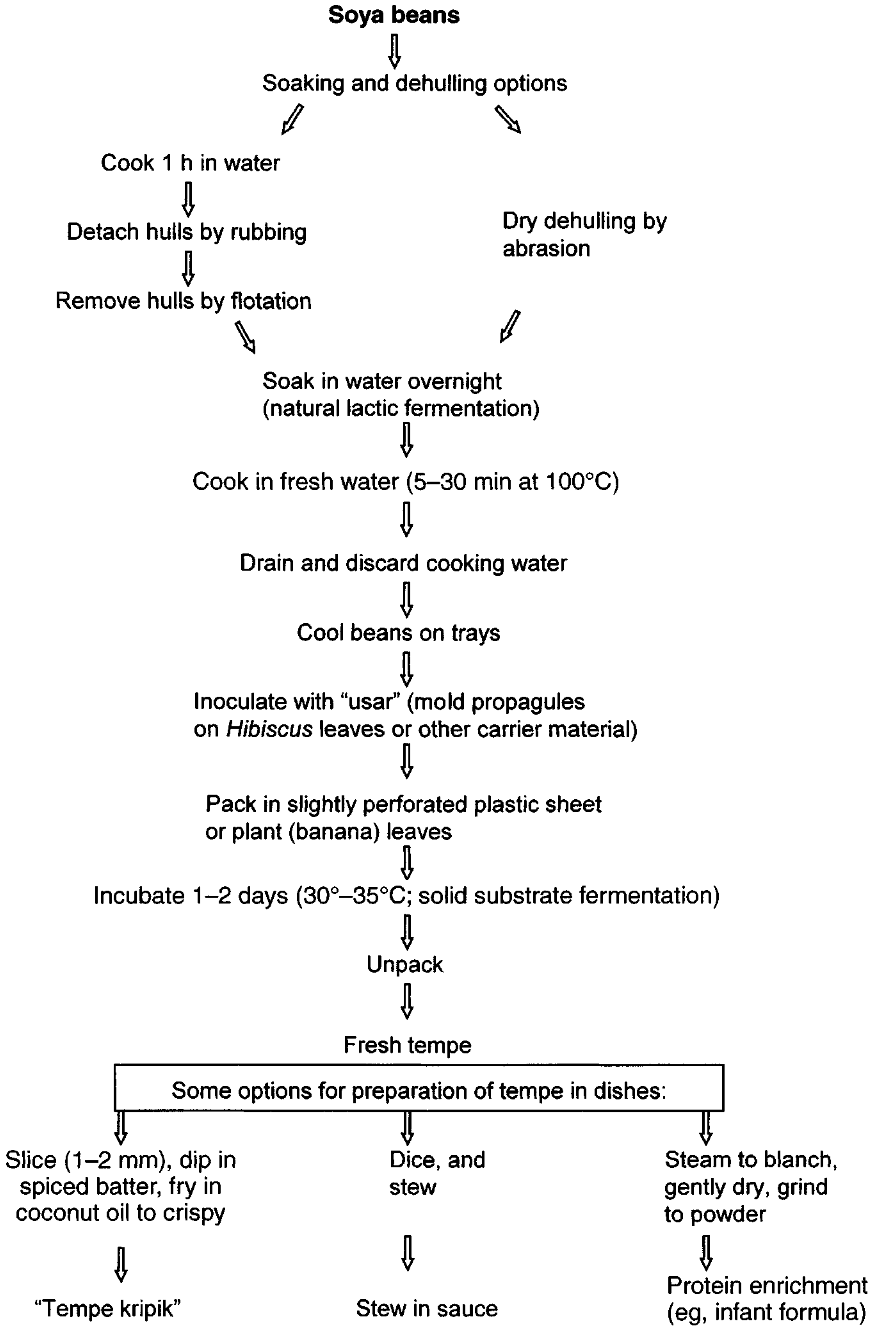
Soya beans
Soaking and dehulling options
Cook 1 h in water
Detach hulls by rubbing
Remove hulls by flotation
Dry dehulling by abrasion
Soak in water overnight (natural lactic fermentation)
Cook in fresh water (5–30 min at 100°C)
Drain and discard cooking water
Cool beans on trays
Inoculate with "usar" (mold propagules on *Hibiscus* leaves or other carrier material)
Pack in slightly perforated plastic sheet or plant (banana) leaves
Incubate 1–2 days (30°–35°C; solid substrate fermentation)
Unpack
Fresh tempe
Some options for preparation of tempe in dishes:
Slice (1–2 mm), dip in spiced batter, fry in coconut oil to crispy
Dice, and stew
Steam to blanch, gently dry, grind to powder
"Tempe kripik"
Stew in sauce
Protein enrichment (eg, infant formula)

Figure 26-14 Flow Scheme of Tempe Process. *Source:* Reprinted with permission from M.J.R. Nout and F.M. Rombouts, Recent Developments in Tempe Research, *Journal of Applied Bacteriology,* Vol. 69, pp. 609–633, © 1990, Blackwell Science, Ltd.

anthranilic acid,[31] which has antioxidative properties. These and similar substances have been associated with free radical scavenging and anti-carcinogenic effects.[31]

Raw soya beans contain several ANFs, including phytate (which forms complexes with minerals, protein, and starch), hemagglutinins or lectins (glycoproteins that can bind with intestinal epithelial cells and cause damage to villi), protease inhibitors including trypsin inhibitors, which affect the digestion of protein, and oligosaccharides such as raffinose and stachyose, which cause flatulence and increase stool passage rates, and thus reduce nutrient absorption. Some of these ANFs, such as phytate, are heat-stable

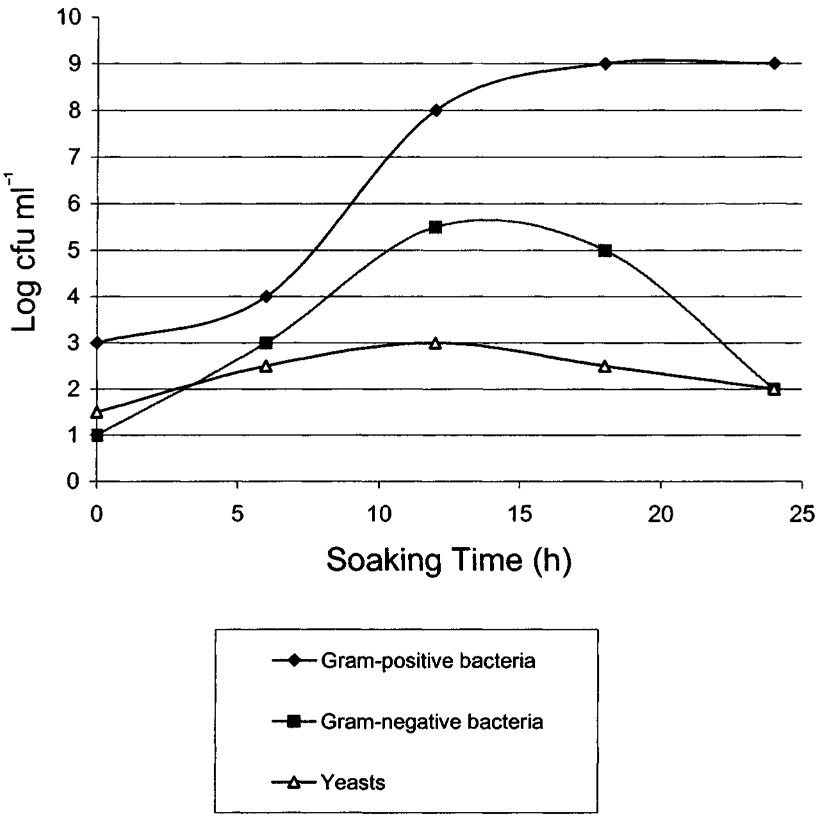

Figure 26-15 Microbiological Changes during Soya Bean Soaking Stage of Tempe Process. *Source:* Data from R.K. Mulyowidarso et al., The Microbial Ecology of Soybean Soaking for Tempe Production, *International Journal of Food Microbiology*, Vol. 8, pp. 35–46, © 1989.

but degradable by fermentation. Others such as Kunitz' trypsin inhibitor, are heat-sensitive but are not easily degraded by fermentation microorganisms.

Processing of soya beans for maximum nutritional benefit should therefore include a combination of treatments, such as grinding, soaking, and cooking (enabling an enzymic degradation prior to heat inactivation), or moistening, cooking, and fermentation.

Prevention of Spoilage

Spoilage during fermentation may occur if beans were not adequately acidified or cooked prior to inoculation. The neutral pH of the cooked beans enables rapid growth of *Bacillus* spp. as well as Enterobactericeae.[100] Poor quality inoculum (ie, poor viability of the mold spores, heavy contamination with other molds and spoilage bacteria) allows outgrowth of undesirable microorganisms.

Table 26-20 gives an indication of the microbial growth in fresh, laboratory-made soya bean tempe when stored at various temperatures. The initial values for total aerobic mesophilic bacteria are considerably lower than those found in commercial tempe (Table 26-18) because these were fresh, and relatively "clean" tempe samples, obtained using pure culture mold starter, using acidified beans, and employing hygienic methods. Such beans have approximately pH 5, and a water activity close to 1.0. Even under such mildly acidic conditions, bacteria in the soaked beans will survive the cooking treatment, and some will be introduced during the subsequent handling of the product. During storage, they will proliferate depending on inhibitory effects such as competition by lactic acid bacteria or inhibition by low temperature, and by acid. The latter effect is counteracted by the pH increase caused by NH_3 formation from protein degradation. This also explains why in commercial tempe sometimes rather high counts of objectionable bacteria are encountered. The product itself is a good growth medium for such bacteria; adequate hygiene and the competition caused by a vigorously active starter of molds and lactic acid bacteria in combination with suitably low storage temperatures are essential for safe storage. Traditionally in Indonesia, fresh tempe is not stored for longer than a few days. In the Netherlands,

Table 26-18 Microbiological Composition of Commercial Tempe Made in the Netherlands (*n* = 110 samples from different producers; production methods not specified)

	Log cfu g^{-1} (in % of all samples)
Aerobic mesophilic bacteria	>7.0 (98)
Enterobacteriaceae	>5.0 (67)
Lactic acid bacteria	>7.0 (81)
Staphylococcus aureus	>5.0 (13)
Bacillus cereus	>5.0 (11)
Escherichia coli	>5.0 (3)

Major Molds	*% of 50 Isolates*	*Major Yeasts*	*% of 111 Isolates*
Rhizopus oryzae	30	*Trichosporon beigelii*	30
Rhizopus oligosporus	20	*Clavispora lusitaniae*	15
Mucor indicus	28	*Candida maltosa*	12
Mucor circinelloides	8	*Candida intermedia*	10
Geotrichum candidum	8	*Yarrowia lipolytica*	6

Source: Adapted with permission from R.A. Samson, J.A. Van Kooij, and E. De Boer, Microbiological Quality of Commercial Tempeh in the Netherlands, *Journal of Food Protection*, Vol. 50, pp. 92–94, © 1987, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

most commercial fresh tempe is wrapped in polyethylene film and kept refrigerated. Under such conditions, the product can be stored for a few weeks before it spoils (turning sour, musty, or smelly). Alternatively, tempe may be pasteurized in a suitable foil package. These pasteurized tempe and derived products (spreads, salads) can be stored refrigerated for several months. No microbiological data are available for these pasteurized products.

Table 26-19 Chemical Changes Resulting from Tempe Fermentation (representing market samples of commercial traditional tempe, as well as laboratory-made experimental tempe)

	100 g Cooked Soya Bean Cotyledons Dry Matter	*100 g Tempe Dry Matter*
Crude protein (N * 5.7) (g)	48.3	48.6–49.7
Crude fat (g)	25.7–28.6	25.2–26.3
Carbohydrates (g)	2.0–2.8	1.9–2.2
Insoluble fiber (g)	13.7–14.3	11.1–12.9
Soluble fiber (g)	4.0–5.0	3.2–8.2
Phytic acid (g)	1.0–1.2	0.2–0.4
Thiamine B1 (mg)	0.30–0.48	0.28–0.30
Riboflavin B2 (mg)	0.10–0.15	0.50–0.65
Nicotinic acid (mg)	0.50–0.67	2.52–3.00
Pantothenic acid (μg)	430–500	520–1000
Pyridoxin B6 (μg)	180–400	830–2000
Cyanocobalamin B12	0.15 (ng)	0.7–8 (μg)

Source: Data from M.J.R. Nout and F.M. Rombouts, Recent Developments in Tempe Research, *Journal of Applied Bacteriology*, Vol. 69, pp. 609–633, © 1990, and W.B. Van der Riet et al., Food Chemical Analysis of Tempeh from South African–Grown Soybeans, *Food Chem*, Vol. 25, pp. 197–206, © 1987.

Control of Safety

The reduction of pH in the soaking water and the beans caused by the lactic fermentation during the soaking stage tends to inhibit any pathogenic bacteria that may be present. During the subsequent mold fermentation (tempe fermentation) challenge experiments with *Bacillus cereus*,[102] *Staphylococcus aureus*,[103] and *Listeria monocytogenes*[6] indicate that the best protection is offered by the combination of acidified beans (pH approx. 5) and the presence of live lactic acid bacteria in the cooked beans at the moment of inoculation with mold spores. For instance, the addition of *L. plantarum* (7 log cfu ml^{-1} soak water) at the soaking stage prevented growth by 5 log cfu g^{-1} *Bacillus cereus* added to subsequently cooked beans[102]; *L. plantarum* at 7 log cfu g^{-1} cooked beans could not inhibit growth of 3 log cfu g^{-1} *Staphylococcus aureus* but no staphylococcal enterotoxins could be formed[103]; and *L. plantarum* at 3.3 log cfu g^{-1} beans fully inhibited the growth of *Listeria monocytogenes* added at 2.5 log cfu g^{-1} beans.[6] Concluding, lactic acid bacteria not only play a role as flavor makers, but they are important in terms of microbiological safety of the product. It is, however, important that in common practice tempe is either fried or stewed before consumption (Figure 26-14).

26.4.3 Bacterial Alkaline Fermentation To Produce Kinema

An unusual food fermentation is caused by *Bacillus* spp., notably *B. subtilis*, *B. licheniformis*, *B. polymyxa*, and *B. natto* in high protein food plants, forming products such as "dawadawa," "natto," and "kinema." In these "alkaline fermentations" a significant pH increase occurs due to protein degradation. Deamination reactions lead to the formation of volatile amines and ammonia, which cause a pungent odor, and cause the pH to increase from approximately 6.0 in the raw material to 8.5 or even 9.0 in the fermented product.

Method of Production

The traditional process for preparation of kinema is outlined in Figure 26-16. Soya beans are cleaned, washed, soaked, cooked, crushed to form a mass containing the seed hulls, wrapped, and fermented in a warm place until a desirable ammoniacal odor, and a stringy texture has been achieved. Kinema is used as a meat substitute and is fried in oil prior to consumption.

In the traditional process, no starter culture is added. Presumably, *Bacillus* spores survive the cooking and the mass is also "inoculated" by the utensils and wrapping materials used.

Conditions for optimizing the traditional process by control of soaking, cooking, wrapping, and fermentation, and the addition of a starter culture of *Bacillus subtilis* have been suggested[139] but this process is not yet applied on a commercial scale.

Microbiological and Chemical Changes during Fermentation

Of 10 kinema samples collected at market sites all contained (per g wet wt of sample) predominantly *Bacillus subtilis* (8.4–8.7 log cfu) and *Enterococcus faecium* (7.4–8.1 log cfu). Seven samples contained *Candida parapsilosis* (<5.2 log cfu) and four contained *Geotrichum candidum* (<4.9 log cfu).[141] Only the *Bacillus* species were detectable on the raw soya beans; *B. subtilis* grows rapidly at 37° and 45°C in cooked soya beans (Figure 26-17).

E. faecium also grows well at 37°C, but *C. parapsilosis* grows only to a limited extent. When tested individually and in mixed cultures, products fermented by the addition of *B. subtilis* were preferred by a sensory panel to those fermented by combinations of *B. subtilis*, *E. faecium*, *C. parapsilosis*, and *G. candidum* or by *E. faecium* alone.[139]

Table 26-21 shows the approximate composition of soya beans and kinema. Whereas the nitrogen content (approximately 8.5% of dry matter) does not change significantly, proteins are degraded (decrease of protein-nitrogen from approximately 7.5% to 6% of dry matter), causing significant increases of water-soluble-N (from 1% to 5% of dry matter) and non-protein-N (NPN) (from 1% to 2.5% of dry matter). Theoretically, protein-N and NPN should add up to total N, but some protein-N may also be measured as water-soluble-N.

During fermentation, fats (glycerides) are degraded, resulting in a significant increase of free fatty acids (FFA). Part of the released FFA is assimilated by the microorganisms as a source of carbon and energy. Ash content is significantly higher in some samples of kinema; this is explained by the local custom of mixing some wood ash into the product for unknown reasons. The increase of the pH is not always very high; probably it depends on the strains of microorganisms dominating the fermentation, and their ability to produce ammonia.

Inactivation of the antinutritional factors of soya beans, discussed in Section 26.4.2, is important during production of kinema and takes place during the cooking stage.

Table 26-20 Microbial Growth during Storage of Tempe

Log cfu g^{-1}	*Fresh Tempe*	*1 Week 3°–4°C*	*2 Weeks 3°–4°C*	*1 Week 10°C*	*3 Days 21°C*
Total aerobic mesophilic bacteria	5.60	5.60	5.60	7.20	7.90
Bacterial spores	2.11	2.11	2.11	3.80	4.20
Psychrotrophic bacteria[a]	ND[b]	5.62	8.70	ND	ND

[a] Psychrotrophic bacteria were dominated by *Pseudomonas*, Enterobacteriaceae, and enterococci.

[b] ND = Not Determined.

Source: Data from M. Ashenafi, Microbiological Evaluation of Tofu and Tempeh during Processing and Storage, *Plant Foods for Human Nutrition*, Vol. 45, pp. 183–189, © 1994, and M.J.R. Nout et al., The Influence of Some Process Variables and Storage Conditions on the Quality and Shelf-Life of Soybean Tempeh, *Antonie van Leeuwenhoek*, Vol. 51, pp. 532–534, © 1985.

Prevention of Spoilage

Kinema is preserved usually by dehydration. At the conclusion of the traditional process, the fermented product is sun dried for 1 or 2 days in order to reduce the moisture content to 9% to 12%. During this period, the product darkens considerably due to polyphenoloxidase activity.[141] Other alkaline fermented products, such as dawadawa, are dried in a similar way.[110] By adequate dehydration, the shelf life of kinema and similar products is extended to approximately 1 year.[110,141]

Salt has also been added to dawadawa.[110] NaCl 5% w/w inhibited *B. subtilis* growth and proteolytic activity and enabled the fermentation and enzymatic degradation to be arrested at the desired level.

Control of Safety

Alkaline fermentations are unusual not only for their change of pH, but also for the bacteria involved in the fermentation. *Bacillus subtilis* and *B. licheniformis* are known to be associated with food poisoning (see Chapter 39). At present, no data are available on the ability of the *Bacillus* spp. occurring in alkaline fermented foods, to produce toxins. Considering that these products are traditionally consumed, one might assume that the conditions during production, preparation, and consumption lead to safe products. Presumably, either people who eat kinema, for example, have become resistant to these bacteria, or they are lucky that the predominating strains differ from those causing food poisoning. Alternatively, frying or other preparatory conditions may ensure the safety of this type of product.

Other main concerns are mycotoxins, bacterial pathogens, and toxic metabolites. No published data are available concerning kinema, but some relevant information is available about the similar product dawadawa.

The opportunities for molds to grow on dawadawa during or after the fermentation and sun drying are very limited. *A. flavus* was inoculated on dawadawa, and its aqueous extract, and was unable to grow on both products.[4] Dawadawa itself has a low water activity that may have caused the inability of *A. flavus* to grow on it. However, the inability of *A. flavus* to grow in a watery suspension of dawadawa was overcome after neutralizing its high pH.[110] This would indicate that the risks of aflatoxin accumulation during fermentation or storage are low. On the other hand, no data are available on aflatoxin levels in the raw material or on the effect of the fermentation on aflatoxins. In this type of product, in the presence of

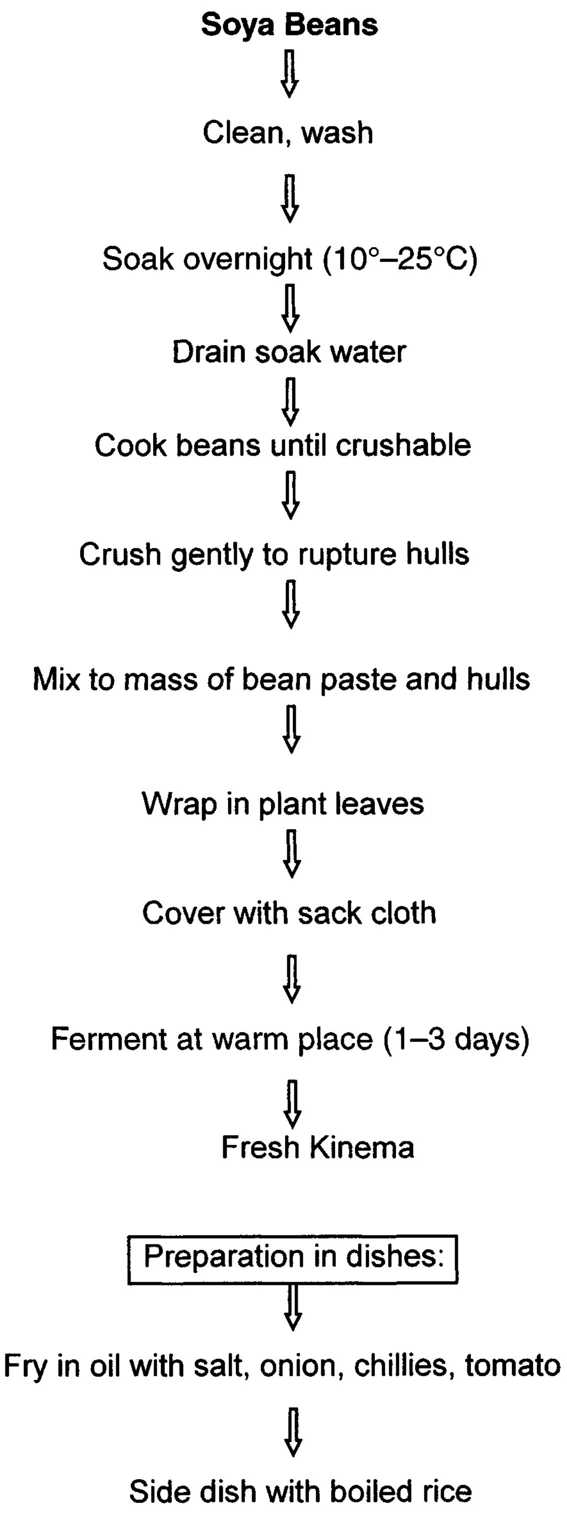
Soya Beans
Clean, wash
Soak overnight (10°–25°C)
Drain soak water
Cook beans until crushable
Crush gently to rupture hulls
Mix to mass of bean paste and hulls
Wrap in plant leaves
Cover with sack cloth
Ferment at warm place (1–3 days)
Fresh Kinema
Preparation in dishes:
Fry in oil with salt, onion, chillies, tomato
Side dish with boiled rice

Figure 26-16 Traditional Process for Production of Kinema. *Source:* Reprinted with permission from P.K. Sarkar, J.P. Tamang, P.E. Cook, and J.D. Owens, Kinema—A Traditional Soybean Fermented Food: Proximate Composition and Microflora. *Food Microbiology,* Vol. 11, pp. 47–55, © 1994, Academic Press Ltd.

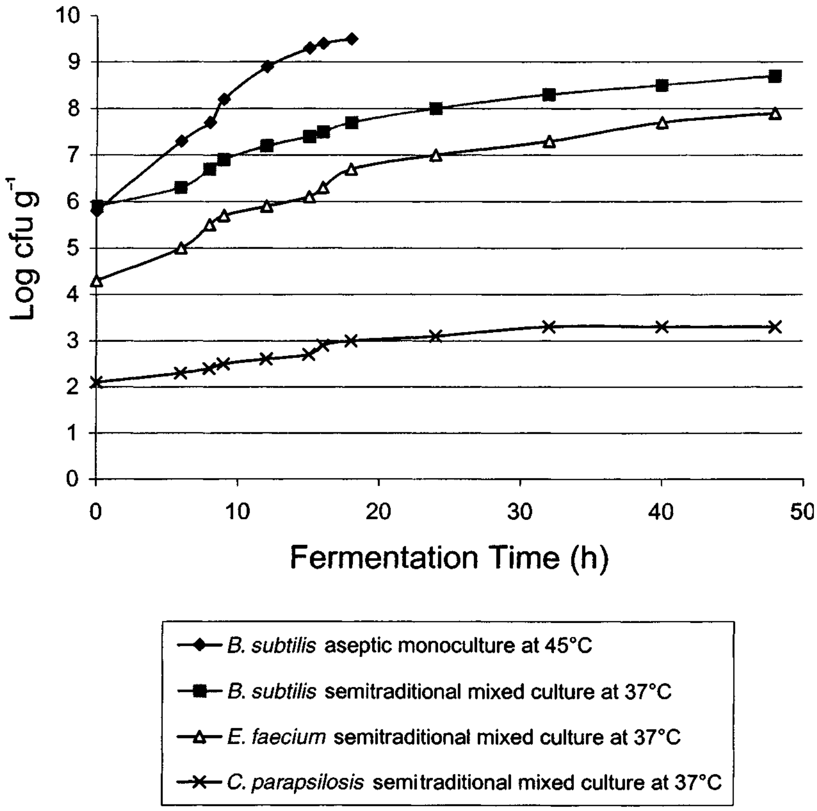

Figure 26-17 Microbial Growth in Semitraditional Mixed Culture Kinema (37°C) and Kinema Produced under Aseptic Conditions with a Monoculture of *B. subtilis* (45°C) (log cfu g^{-1}). *Source:* Reprinted with permission from P.K. Sarkar and J.P. Tamang, Changes in the Microbial Profile and Proximate Composition during Natural and Controlled Fermentations of Soybeans To Produce Kinema, *Food Microbiology,* Vol. 12, pp. 317–325, © 1995, Academic Press Ltd.

significant levels of ammonia, part of the aflatoxins that may be present would probably be degraded, as was demonstrated in ammonia-treated maize.[163] No data are available about other mycotoxins.

There appear to be no published studies of the incidence of human pathogenic bacteria in kinema and similar products. The bacterial hygiene and safety of African alkaline fermented foods have been termed questionable[156]; however, the reduced water activity and the salt in this type of product would inhibit *Clostridium* and *Salmonella* spp.[110] Pertinent data will be needed on bacterial quality of commercial samples, and on the limitation of growth and toxin production by the product.

Another area of concern is the formation of biogenic amines including tyramine, histamine, putrescine, and cadaverine.[156] Studies of the concentrations of these compounds in kinema and similar products have not been made. The presence of high levels of precursor amino acids increases the chance of biogenic amine formation if decarboxylase-active bacteria are present. The safety of alkaline fermented proteinaceous foods could certainly be improved by using starter cultures that have been selected on criteria including nonpathogenicity and inability to decarboxylate amino acids.

26.4.4 Mixed Fungal, Bacterial, and Yeast Fermentation To Produce Soya Sauce

Method of Production

Soya sauces are of Oriental origin; a wide range of products and processes has been described to produce these flavorings.[45] Generally, Japanese soya sauces ("shoyu") are made of a mixture of equal weights of wheat and yellow-skinned soya beans. However, in Indonesia, soya sauce ("kecap") is made of black-skinned soya beans only. Figure 26-18 outlines the principle of a process for Japanese koikuchi-shoyu, one of the most popular types. Autoclaved soya beans and roasted and crushed wheat are blended and inoculated with a defined starter of *Aspergillus sojae*

Table 26-21 Approximate Composition of Market Samples of Kinema and of Soya Beans

	Soya Beans	*Kinema*
Moisture % w/w	9.5–12.0	57.3–64.8
Crude protein % w/w dm[a]	45.8–48.2	45.8–49.2
Crude fat % w/w dm	20.3–24.0	14.4–19.2
Carbohydrates % (by difference)	22.6–29.4	23.4–33.7
Ash % w/w dm	4.5–5.2	5.8–8.4
pH	6.60–6.85	6.95–8.50
TTA % w/w as lactic	0.01	0.06–0.12
FFA % w/w as linoleic	0.04–0.15	2.1–3.3
Energy MJ/100 g dm	2.0–2.3	1.8–2.1

[a] dm = dry matter.

Source: Reprinted with permission from P.K. Sarkar, J.P. Tamang, P.E. Cook, and J.D. Owens, Kinema—A Traditional Soybean Fermented Food: Proximate Composition and Microflora, *Food Microbiology*, Vol. 11, pp. 47–55, © 1994, Academic Press Ltd.

and *A. oryzae* conidia. The resulting mass is incubated for several days at 25° to 30°C to undergo a solid-substrate fermentation. The resulting product is called "koji" (in Indonesia: "bungkil").

The second phase of the process is a liquid fermentation of the koji in a brine at 16% to 19% w/v NaCl. This stage of liquid fermentation is known under the names "moromi" (Japan) and "baceman" (Indonesia). The fermentation, traditionally carried out at approximately 25° to 30°C, takes several months to complete. During this period, soya beans are solubilized into a hydrolysate and fermentation by halotolerant lactic acid bacteria (*Tetragenococcus halophila*) and osmotolerant yeasts (*Zygosaccharomyces rouxii* and *Candida* spp.) takes place.

The final phase of the process involves filtration of the liquid hydrolysate, bottling, and pasteurization. In Indonesia, sweetened types of kecap are made by adding palm or cane sugar and various herbs and spices to the crude kecap.

Microbiological and Chemical Changes during Fermentation

The Initial Fungal Fermentation Stage. *Aspergillus oryzae* and *A. sojae* are considered to be biovars of *Aspergillus flavus*. Raw materials (soaked and cooked soya beans, either alone or mixed with steamed rice, or with roasted and crushed wheat or barley) are inoculated with about 1% of conidial starter and spread in thin (2 to 5 cm) layers on trays. Some large-scale enterprises for soya sauce production have mechanized this solid-substrate fermentation using reactors with forced ventilation and intermittent agitation. The fungal growth takes a few days at 25° to 30°C and is aimed at maximizing the activity of proteolytic, saccharolytic, and other enzymes in the fermented mass. The koji is usually harvested when spore pigmentation starts.

The solid-substrate fermentation (SSF) takes several days; during this period active mycelial growth and sporulation take place. During this period, *Aspergillus oryzae* and *A. sojae* consume 15% to 30% of the carbohydrates of the blend. Also, CO_2 and heat are produced, and the moisture content of the blend (koji) decreases to 20% to 25%. Most importantly, a variety of proteolytic, saccharolytic, lipolytic enzymes as well as phosphatases and other enzymes are produced. The production, stability, and activity of these enzymes are influenced by the temperature, pH, and water activity of their environment.

The Brine Fermentation Stage. The next step is a submerged fermentation. The salt solution is mixed with the koji and the resulting suspension (moromi) has 16% to 19% w/v NaCl. This suspension will be aged for 6 to 12 months, and during this period a gradual degradation of the macromolecular substrate will take place, caused by the fungal enzymes in the koji. Polysaccharides are degraded into oligo- and monosaccharides, proteins into peptides and free amino acids, and lipids into glycerol and free fatty acids. The salt concentration is crucial and is adjusted depending on the prevailing temperatures. In principle, the salt concentration must be increased at higher temperatures in order to avoid microbial spoilage. Low concentrations of salt will allow rapid enzymatic degradation of substrate, but they will also enable extensive (undesirable) microbial growth. Higher salt concentrations inhibit the activity of lactic acid bacteria (as evidenced by slower pH decrease and inhibition of flavor production) and slow down the enzymatic degradation process, but increase safety from microbial pathogens and toxigenic microorganisms.

During traditional brine fermentation in Japanese soya sauce, a natural succession of halotolerant lactic acid bacteria (*Tetragenococcus halophila*, formerly known as *Pediococcus halophilus*) and osmotolerant yeasts (*Candida* spp. in Indonesian kecap; *Zygosaccharomyces rouxii* in Japanese shoyu) will take place without any inoculation.

In Indonesian soya sauce, the situation may be somewhat different. Figure 26-19 shows that the development of lactic acid bacteria and yeasts is rather simultaneous and the latter are numerically dominated by the lactic acid bacteria. In the situation cited in Figure 26-19, *Zygosaccharomyces rouxii* was not detected.

In larger-scale industrial settings with strict management of hygiene, natural inoculation may not be

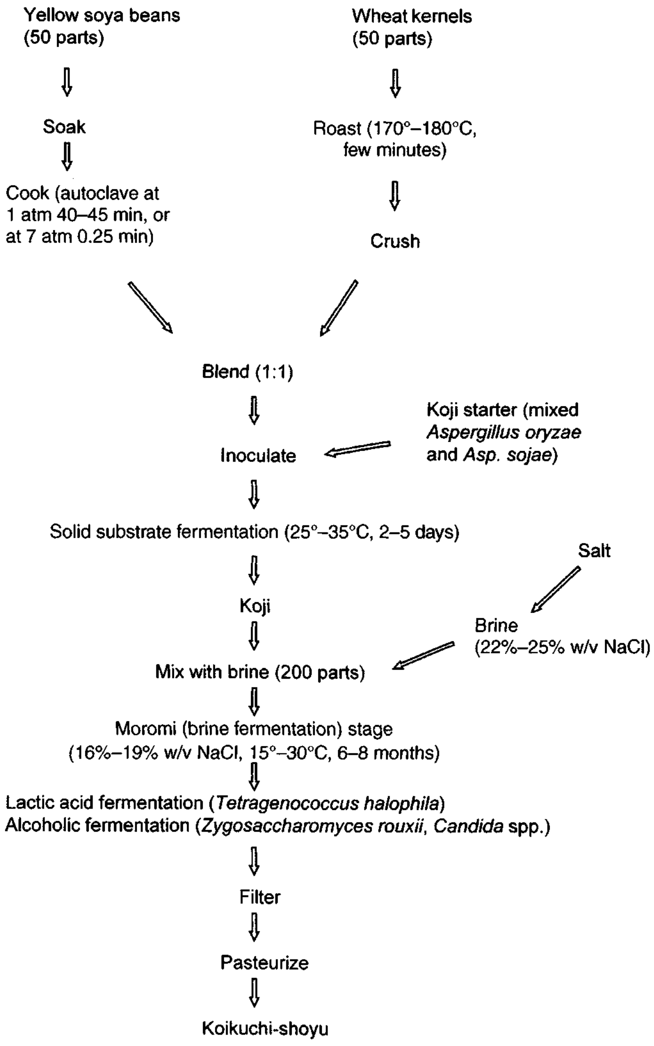
Yellow soya beans
(50 parts)
Wheat kernels
(50 parts)
Soak
Roast (170°–180°C, few minutes)
Cook (autoclave at 1 atm 40–45 min, or at 7 atm 0.25 min)
Crush
Blend (1:1)
Koji starter (mixed *Aspergillus oryzae* and *Asp. sojae*)
Inoculate
Solid substrate fermentation (25°–35°C, 2–5 days)
Salt
Koji
Brine (22%–25% w/v NaCl)
Mix with brine (200 parts)
Moromi (brine fermentation) stage
(16%–19% w/v NaCl, 15°–30°C, 6–8 months)
Lactic acid fermentation (*Tetragenococcus halophila*)
Alcoholic fermentation (*Zygosaccharomyces rouxii*, *Candida* spp.)
Filter
Pasteurize
Koikuchi-shoyu

Figure 26-18 Principle of the Production Scheme of Japanese-Style Soya Sauce (Koikuchi-Shoyu). *Source:* Reprinted with permission from D. Fukushima, Industrialization of Fermented Soy Sauce Production Centering around Japanese Shoyu, in *Industrialization of Indigenous Fermented Foods,* K.H. Steinkraus, ed., pp. 1–88, © 1989, by courtesy of Marcel Dekker, Inc.

sufficient to achieve the desired lactic and alcoholic fermentation. Industrial soya sauce fermentations are therefore inoculated at the required moment with separately grown pure cultures of lactic acid bacteria and yeasts. *T. halophila* is added first to the moromi mash and this is kept at 15°C for 1 month allowing the pH to decrease from 6.5 to 5.0. At this stage, *Z. rouxii* and *Candida* spp. are added, and the temperature is allowed to rise gradually to 28°C during a 4-month period enabling an active alcoholic fermentation.[75] As a result, 2% to 3% ethanol and other metabolites are produced, such as higher (C_{3-5}) alcohols and glycerol. Organoleptic properties are associated in part with levels of alcohol, salt, reducing sugar, color, soluble nitrogen, glutamic acid, titratable acid, and pH. More than 300 compounds have been identified in soya sauce, many of which may contribute to the flavor of the product.

Prevention of Spoilage

Particularly during the brine fermentation stage, the control of pH, dissolved oxygen, and salt concentration is important. During the early part (first 6 days) of the brine fermentation halotolerant lactic acid bacteria reduce the pH and concentration of dissolved oxygen, and the resulting conditions prevent the growth of aerobic spoilage microflora. In Indonesian kecap, such aerobic microorganisms were mainly coryneform bacteria. If aerobic spoilage does occur it takes the form of pellicle formation, and is often associated with the formation of off-odors caused by oxidation.[128]

In later stages of the process (after 6 days of brine fermentation), the combination of low levels of dissolved oxygen, pH <5.4, and NaCl concentration >10% w/v is associated with low levels of corynebacteria and absence of pellicle formation, and facilitates product stability. Long-term shelf life of soya sauce is achieved usually by bottling and pasteurization, and is limited by chemical and sensory changes rather than by microbiological changes. The slightly acidic pH (4.6–4.8), the presence of weak organic acids (mainly lactic, pyroglutamic, acetic, succinic, citric, and formic acids, totaling approx. 1.3% w/v), and considerable NaCl concentrations (16.9% to 19.0% w/v)[45] all contribute to the microbiological stability of the bottled and pasteurized product.

Control of Safety

A. oryzae is regarded as a variety of *Aspergillus flavus*, but, in contrast with *A. flavus*, *A. oryzae* has not been found to produce the highly toxic and carcinogenic aflatoxins. No aflatoxins could be produced by industrial soya sauce starters, and no aflatoxins have ever been found in Japanese soya sauce.[45] In Korean soya sauce, aflatoxins were chemically unstable.[118]

Yeast metabolism of citrulline may lead to the accumulation of carbamic acid, which in turn may react with ethanol to form ethylcarbamate (urethane). The latter compound is toxic, and has been found in several yeast-fermented products, including soya sauce. The levels varied from nondetectable to 35 ppb with a mean of 17.7 ppb; a "no significant risk" level of 0.7 μg day^{-1} has been reported.[80] In view of the widespread daily use of soya sauce in Eastern Asia, process control measures to minimize ethyl carbamate levels will be desirable.

Recently, mutagenic effects of soya sauce were observed using Ames tests.[54] At present, it is not known which substances cause these effects, and whether they are active in human tissue as well.

26.5 CONCLUDING REMARKS

Fermented foods based on vegetables, cereals, and proteinaceous seeds play an important role worldwide, and will continue to do so. The ever-increasing world population will require more affordable nutrients and energy presented in a palatable and safe manner. Fermentation is of interest for reasons of sensory appeal, nutritional and physiological effects in the consumer, and of product safety. However, several cases of foodborne infections and intoxications have been associated with fermented foods.[95] Considering the contamination of food raw materials, the high levels of water activity, nutrients, and pH in some fermented foods, and occasional inadequate processing and storage conditions, a few major risk factors can be identified: (1) the use of previously contaminated raw materials (eg, with mycotoxins, heavy metals, and other stable compounds); (2) lack of pasteurization (such as occurs in some cheese and vegetable products); (3) the use of poorly controlled natural fermentations, or of inadequate fermentation starter cultures (thus providing ineffective substrate colonization, and enabling opportunistic microorganisms to develop); (4) inadequate storage or maturation conditions enabling survival of pathogens, or growth and toxin production (eg, insufficient levels of curing salts; inadequate protection from air exposure); and (5) consumption without prior heating (thus allowing the product to act as a vehicle for foodborne infections, for example).

The study of processes and microbiological and chemical changes taking place during traditional fermentation processes provides the basic data to investigate aspects of safety and nutrition. At the industrial level, but also in the household, consumer safety is a first priority. From a commercial point of

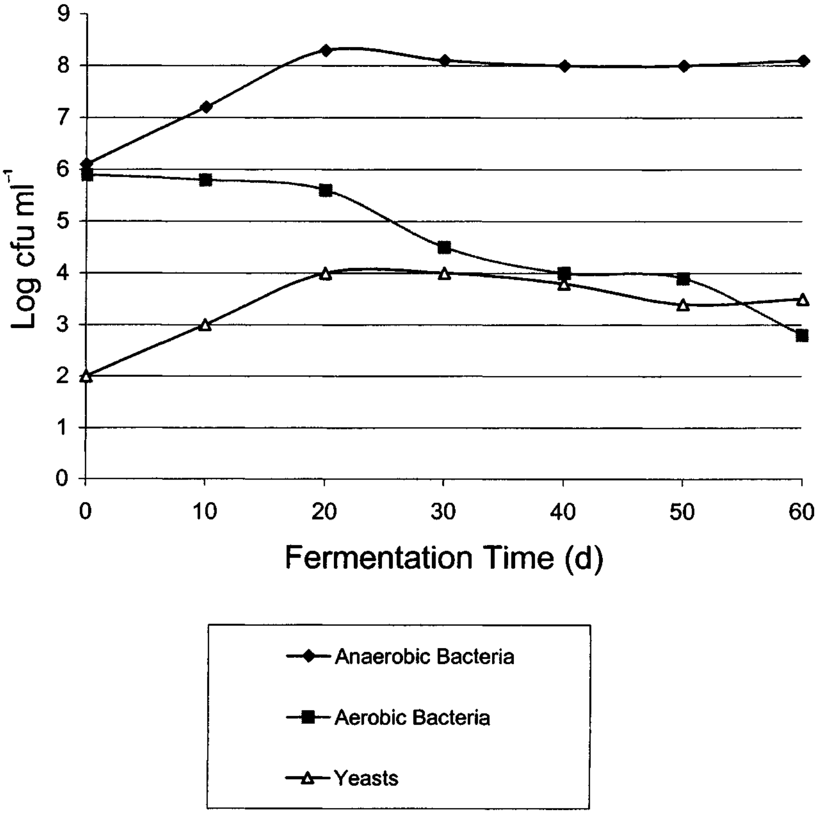

Figure 26-19 Microbiological Changes during the Liquid Fermentation Stage ("baceman" with 15% w/v NaCl concentration) of Indonesian Soya Sauce Fermentation. Anaerobic bacteria: progressively *Tetragenococcus halophila*; aerobic bacteria: coryneform bacteria; yeasts: *Candida* spp. (*C. parapsilosis, C. famata, C. guilliermondii, C. pulcherrima*). *Source:* Data from W.F.M. Röling et al., Changes in Microflora and Biochemical Composition during the Baceman Stage of Traditional Indonesian Kecap (Soy Sauce) Production, *J. Ferm. Bioeng.*, Vol. 77, pp. 62–70, © 1994.

view, control of processes is essential, based on a thorough understanding of the fundamentals.

Future developments are likely to include (1) optimization of fermentation practices to meet food safety requirements, and (2) increased use of (safe) defined cultures of microorganisms and of enzymes that have been tailor-made by molecular-biological techniques. This practice will be especially interesting to achieve effects that cannot be realized with conventional ingredients. For instance, traditional fermented foods (well known and popularly accepted) are increasingly used as a vehicle for the use of probiotic microorganisms. Probiotic fermented dairy products[122] are available commercially; probiotic fermented cereal soups[77] are actively investigated.

From a processing point of view, large-scale industrial manufacture of Japanese soya sauce may provide an indication of what is possible in terms of continuous processes using immobilized microorganisms, membrane filters, solid-substrate bioreactors, and others.[75] Considering the increasing world population, industrialization of (fermented) food manufacture is bound to expand; however, one has to realize that at present, a significant segment of the population derives income from the artisanal processing of foods.

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27 Fresh and Processed Fruits

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27.1 INTRODUCTION

A fruit is defined strictly as the seed-bearing product of a flowering plant (tree, bush, or herb), and is derived from transformation of the ovary or associated tissue. This definition includes cereals, pulses, nuts, and spice fruits, as well as the fleshy fruits, which are considered in this chapter. The definition of fruits includes tomatoes, peppers, cucumbers, and other cucurbits, regarded as vegetables in the culinary context.

The world production figures for fruits (Table 27-1) indicate the relative importance of different fruits; grapes are of major importance but are used mainly to produce wines.

Fruits are classified commonly into temperate zone, subtropical, and tropical products.[59] Temperate zone fruits include pome fruits (eg, apple and pear); stone fruits (eg, apricot, cherry, nectarine, peach, and plum); and soft fruits (eg, grape, strawberry, raspberry, and cranberry). Subtropical fruits include citrus fruits (eg, orange, lemon, and grapefruit), and noncitrus fruits (eg, avocado, fig, and kiwifruit). Tropical fruits include banana, mango, papaya, pineapple, and a range of minor fruits.

The source of these fruits determines the type of plant pathogen that causes disease during growth and that also may cause postharvest spoilage. The growing conditions of fruits also influence the microbial flora on the product and the incidence of human pathogenic microorganisms, which may survive during harvesting and retail sale and cause outbreaks of disease.

27.2 COMPOSITION AND PROPERTIES

The majority of fruits contain between 10% and 25% of carbohydrates, less than 1% of protein, and less than 0.5% fat. Avocados and olives are exceptional in that they contain a relatively high concen-

Table 27-1 World Production of Fruits in 1997

Fruit	*Million Metric Tons*
Tomatoes	88
Oranges	64
Bananas	59
Grapes	58
Apples	56
Watermelons	46
Plantains	30
Mangoes	23
Cantaloupes and other melons	18
Eggplants	17
Mandarins (all types)	17
Peppers/chillies	16
Pumpkins/squashes	14
Pears	13
Pineapples	13
Peaches/nectarines	11
Lemons/limes	9
Plums	8
Papayas	5
Grapefruits	5
Strawberries	3
Avocados	2

Source: Data from Food and Agriculture Organization, *Production Yearbook 1997*, Vol. 51, © 1998, U.N. Food and Agriculture Organization.

tration of fat, between 3% and 30% in avocados and up to 20% in olives.[65] The carbohydrate content of fruits includes cellulose, hemicellulose, lignin, and pectic substances, all of which contribute to the dietary fiber in the human diet. Most fruits are relatively acidic; acids can account for up to 2% to 3% of the total fresh weight of fruits such as lemons and limes. The major acids present in many fruits are malic and citric, but tartaric acid is abundant in most cultivars of grapes, and quinic acid is important in some fruits such as plum, cherry, and kiwifruit. The concentration of acid and the pH in fruit are influenced by the cultivar, the soil conditions, and the maturity of the fruit.[109] Examples of pH values are shown in Table 27-2.

Fresh fruits are an important source of dietary vitamins, particularly vitamins C and A, and of minerals including magnesium, iron, potassium, phosphorus, and calcium.[59]

Fruits can be divided into two groups: climacteric and nonclimacteric. These groups show differences in their pattern of respiration and in their ability to ripen after harvest.[59,63,133]

Table 27-2 Approximate pH and Main Organic Acids of Selected Fruits

Fruit	*pH*	*Main Organic Acids*
Lemon[a]	2.2–2.4	citric
Cranberry[a]	2.5–2.7	citric, malic, quinic
Passionfruit[a]	2.6–3.3	citric, malic
Plum[a]	2.8–4.6	malic, quinic
Grapefruit[a]	2.9–3.4	citric
Grape[a,e]	3.0–4.5	tartaric, malic
Orange[a]	3.0–4.0	citric, malic
Kiwifruit[b]	3.1–4.0	citric, malic, quinic
Peach[a]	3.1–4.2	malic, citric
Cherry[a]	3.2–4.7	malic, quinic, citric, isocitric
Pineapple[a]	3.2–4.0	citric, malic
Strawberry[c,d]	3.3–3.6	citric, malic
Apple[a]	3.3–4.1	malic, citric, lactic
Mango[a]	3.3–3.7	citric, tartaric
Pear[a]	3.4–4.7	malic, citric, quinic
Tomato, under-ripe[e]	3.2–4.5	
Tomato, ripe[e]	3.4–4.7	
Tomato, over-ripe[e]	4.0–4.8	
Banana[a]	4.5–5.2	citric, malic, tartaric
Papaya[a]	4.5–6.0	citric, malic, ketoglutaric
Watermelon[a]	5.8–6.0	citric, malic
Melon, cantaloupe[a]	6.2–6.5	citric, malic
Melon, honeydew[a]	6.3–6.7	

[a] Data from Splittstoesser.[109]
[b] Data from Luh and Wang.[66]
[c] Data from Deuel.[30]
[d] Data from van Gorsel et al.[125]
[e] Data from Patil et al.[81]

Climacteric fruits undergo a sudden surge in their respiration rate, which is associated with ripening, usually after harvest. This increased respiration is triggered by ethylene that is produced by the fruit itself. Because of this response to ethylene, climacteric fruits can be harvested when mature but unripe, and can be ripened off the plant after harvest by ethylene produced by the fruit or by the controlled application of ethylene. Climacteric fruits include tomato, banana, apple, pear, peach, plum, avocado, and kiwifruit.

Nonclimacteric fruits produce very little ethylene and their rate of respiration remains constant (at constant temperature) and may fall slightly after harvest. These fruits do not ripen in response to treatment with ethylene, except that degreening (degradation of chlorophyll) takes place in some fruits. These fruits do not continue the ripening process after removal from the plant, and must be ripened on the plant before harvest. Nonclimacteric fruits include citrus, grape, pineapple, strawberry, cherry, olive, pepper, and cucumber.

The compositional changes that occur during ripening have a marked effect on the susceptibility of fruits to physical damage and to microbial spoilage. During ripening starch is converted into sugars, principally sucrose, glucose, and fructose; the total concentration of sugars can be about 8% in juice of apples, 20% in juice of grapes, and 4% in juice of strawberries.[59] Polysaccharides such as cellulose, hemicellulose, pectin, and lignin, which are present mainly in the cell wall, are broken down into smaller molecular weight, soluble compounds, resulting in softening of the tissue. The acid content of fruits tends to decrease and the pH to increase during ripening because of utilization of organic acids during respiration or their conversion into sugars. As fruits ripen, the characteristic skin color, flavor, and aroma develop and the fruit reaches its stage of prime eating quality or "ripeness." Subsequently, as the rate of respiration declines, senescence sets in, off-flavors develop, and the dying tissues become increasingly susceptible to microbial spoilage.

27.3 MAJOR GROUPS OF MICROORGANISMS ASSOCIATED WITH FRESH FRUITS

As the flower bud develops into a flower and eventually into a mature fruit the surfaces are colonized by a succession of microorganisms. Many of these microbes originate from the soil and may be spread by wind or rain, or transported by insects. The majority of microbes remain on the surface of fruits; occasionally microorganisms, particularly fungi, invade the fruit while it is still on the plant, and the fruit may then rot, lose moisture, and become "mummified." The mummified fruits may remain attached to the plant or fall to the ground, but in either case they tend to give rise to large numbers of fungal spores, which may be disseminated by wind, water, and insects. In suitable weather conditions, infection of grapes with *Botrytis cinerea* results in mummification of the fruits on the vine; the fruits become dried, concentrating sugars and glycerol ("Noble rot"), and these fruits are used to make certain sweet wines.[94]

Low numbers of bacteria have been reported within some fruits and vegetables, in particular tomatoes and cucumbers.[71,97] In tomatoes, the bacteria were found most frequently in the stem scar and central core, and there was evidence that the bacteria entered the fruit in the vicinity of the sepals; this was linked with the use of overhead irrigation.[96] In cucumbers, the bacteria were more abundant in the periphery than in the central axis, suggesting that they may have entered through stomates or broken hairs.[97]

Molds are important contaminants of fruit surfaces but assessment in terms of numbers present is complicated by the presence of extensive mycelia and of fruiting structures containing large numbers of spores. High numbers of yeasts are often present but there are few data on the numbers of bacteria present. Up to 7.8 × 10^5 yeasts g^{-1} on sound, mature apples and up to 1.4 × 10^6 g^{-1} on grapes were reported by Davenport,[25] and counts of 10^3–10^4 yeasts g^{-1} on apples, and 10^7 yeasts g^{-1} on grapes were reported by Splittstoesser.[109] On freshly harvested soft fruit (strawberries, raspberries, and blackberries), the number of yeasts was between 10^4 and 10^6 g^{-1}, that of molds was up to 10^4 g^{-1}, and the number of bacteria was between 10^5 and 10^6 g^{-1}.[28] Fruit that has been damaged by birds, insects, or pathogenic fungi is liable to contain high numbers of yeasts that are able to multiply on the damaged fruit tissue. The numbers of lactic acid bacteria on fruit as received from orchards and vineyards is usually very low.[109] In a study of sound grapes, about 50% of samples were contaminated with acetic acid bacteria in numbers of 10–10^4 g^{-1}; all the strains isolated were *Gluconobacter suboxydans*.[110]

The numbers and types of microorganisms present on freshly harvested fruit will depend on the weather, the season, the time of harvest within a season, the type of fruit and its proximity to the ground, irrigation, and preharvest treatment with chemicals such as fungicides.

Where bacteria pathogenic to humans are present on fruits, this is predominantly a result of contamination from animals and humans. Wild animals and birds can be a source of contamination but information on outbreaks of disease in humans associated with the consumption of fruits and fruit products indicates that contamination has often resulted from exposure of fruit to human or animal waste or to irrigation water or other water that has been contaminated from these sources.

27.4 MICROBIOLOGICAL SPOILAGE OF FRESH FRUITS

Postharvest spoilage of fresh fruits is often caused by plant-pathogenic microorganisms that cause disease in the field. Some disease organisms cause local disfigurement in the form of discrete ***spots, cankers,*** or ***scabs,*** which may develop no further after harvest. More often, however, the fruit is harvested in apparently good condition, and subsequent deterioration, once started, tends to be progressive. The same organism can cause a ***dry rot*** under conditions of moderate humidity or a ***wet rot*** under conditions of high humidity. The term "mildew" is sometimes used loosely to describe any superficial mold growth. Strictly speaking, ***downy mildews*** are caused by fungi of the family Peronosporaceae, while ***powdery mildews*** are caused by fungi belonging to the Erysiphaceae. ***Anthracnose*** means "coal-like," and was first used to describe a disease of grapes in which blackening of the tissues was a striking feature. It is now used for a group of diseases, characterized by black lesions that become covered with pink spore masses. The causal fungi are certain species in the order Melanconiales.

The major causes of postharvest spoilage (postharvest disease) in fruits are shown Table 27-3. The main spoilage organisms of a given fruit may be very dependent on the growing area.

Postharvest spoilage can occur as a result of infection in the field. For example, branches of mango trees may be permeated by endophytic mycelium of *Dothiorella dominicana,* which subsequently can result in a postharvest stem-end rot of the fruit.[57] Other microorganisms may remain on the surface of the fruit, fungal spores may germinate but the organism may fail to penetrate the fruit, or penetration into the fruit may occur but the organism may fail to progress into the tissue (remain quiescent) for a period of time, often until ripening occurs.[88] Some

Table 27-3 Principal Postharvest Diseases of Fruits of Commerce

	Pathogen	
	Sexual State[a]	*Asexual State*[b]
	TEMPERATE ZONE FRUITS	
Pome fruits (apples, pears)		
Gray mold rot	*Botryotinia fuckeliana*	*Botrytis cinerea*
Blue mold rot	—	*Penicillium expansum*
Brown rot	*Monilinia* spp.	*Monilia* spp.
Bull's eye rot	*Pezicula malicorticis*	*Gloeosporium perennans*
Ripe spot	*Pezicula alba*	*Gloeosporium album*
Alternaria rot	—	*Alternaria alternata*
Eye rot	*Nectria galligena*	*Cylindrocarpon mali*
Mucor rot	*Mucor piriformis*	*Mucor piriformis*
Phytophthora rot	*Phytophthora* spp.	*Phytophthora* spp.
Stone fruits (peaches, nectarines, apricots, plums, cherries)		
Brown rot	*Monilinia* spp.	*Monilia* spp.
Gray mold rot	*Botryotinia fuckeliana*	*Botrytis cinerea*
Blue mold rot	—	*Penicillium expansum*
Rhizopus rot	*Rhizopus* spp.	*Rhizopus* spp.
Cladosporium rot	—	*Cladosporium herbarum*
Soft fruits (strawberries, raspberries)		
Gray mold rot	*Botryotinia fuckeliana*	*Botrytis cinerea*
Leak	*Rhizopus stolonifer*	*Rhizopus stolonifer*
Leak	*Mucor piriformis* spp.	*Mucor piriformis* spp.
Anthracnose	*Glomerella cingulata*	*Colletotrichum* spp.
Yeasty rot	*Saccharomyces* spp.	*Saccharomyces* spp.
Grapes		
Gray mold rot	*Botryotinia fuckeliana*	*Botrytis cinerea*
Blue mold rot	—	*Penicillium* spp.
Black mold rot	—	*Aspergillus niger*
Rhizopus rot	*Rhizopus* spp.	*Rhizopus* spp.
Sour rot	—	*Acetobacter* spp. and *Saccharomyces* spp.

continued

Table 27-3 Continued

	Pathogen	
	Sexual State[a]	*Asexual State*[b]
	SUBTROPICAL FRUITS	
Citrus fruits (oranges, lemons, limes, mandarins, grapefruits)		
Green mold rot	—	*Penicillium digitatum*
Blue mold rot	—	*Penicillium italicum*
Stem-end rot	*Physalospora rhodina*	*Botryodiplodia theobromae*
Stem-end rot	*Diaporthe citri*	*Phomopsis citri*
Alternaria rot	—	*Alternaria citri*
Sour rot	—	*Geotrichum candidum*
Brown rot	*Phytophthora* spp.	*Phytophthora* spp.
Avocados		
Anthracnose	*Glomerella cingulata*	*Colletotrichum gloeosporioides*
Stem-end rot	*Botryosphaeria ribis*	*Dothiorella gregaria*
Stem-end rot	—	*Phomopsis perseae*
Kiwifruits		
Gray mold rot	*Botryotinia fuckeliana*	*Botrytis cinerea*
Blue mold rot	—	*Penicillium* spp.
Ripe rot	*Diaporthe actidiniae*	*Phomopsis actidiniae*
Melons		
Alternaria rot	—	*Alternaria alternata*
Bacterial soft rot	—	*Erwinia carotovora*
Charcoal rot	—	*Macrophomina phaseolina*
Cladosporium rot	—	*Cladosporium herbarum*
Fusarium rot	—	*Fusarium* spp.
Gray mold rot	*Botryotinia fuckeliana*	*Botrytis cinerea*
Pink mold rot	—	*Trichothecium roseum*
Tomatoes		
Alternaria rot	—	*Alternaria alternata*
Bacterial soft rot	—	*Erwinia carotovora*
Cladosporium rot	—	*Cladosporium herbarum*
Late blight rot	*Phytophthora infestans*	*Phytophthora infestans*
Black rot	*Didymella lycopersici*	*Phoma lycopsersici*
Southern blight	*Corticium rolfsii*	*Sclerotium rolfsii*
Sour rot	—	*Geotrichum candidum*
	TROPICAL FRUITS	
Bananas		
Crown rot	—	*Fusarium pallidoroseum*
Crown rot	*Ceratocystis paradoxa*	*Thielaviopsis paradoxa*
Anthracnose	—	*Colletotrichum musae*
Finger rot	—	*Botryodiplodia theobromae*
Cigar-end rot	—	*Verticillium theobromae*
Mangoes		
Anthracnose	*Glomerella cingulata*	*Colletotrichum gloeosporioides*
Stem-end rot	—	*Botryodiplodia theobromae*
Stem-end rot	*Botryosphaeria ribis*	*Dothiorella gregaria*
Black mold rot	—	*Aspergillus niger*
Bacterial black speck	—	*Xanthomonas campestris*

continued

Table 27-3 Continued

	Pathogen	
	Sexual State[a]	*Asexual State*[b]
Papayas		
Anthracnose	*Glomerella cingulata*	*Colletotrichum gloeosporioides*
Black rot	*Mycosphaerella caricae*	*Phoma cariae-papayae*
Phytophthora rot	*Phytophthora palmivora*	*Phytophthora palmivora*
Rhizopus rot	*Rhizopus stolonifer*	*Rhizopus stolonifer*
Pineapples		
Black rot	*Ceratocystis paradoxa*	*Thielaviopsis paradoxa*
Fruitlet core rot	—	*Fusarium moniliforme*, *Penicillium funiculosum*
Pink disease	—	*Acetobacter* spp., *Enterobacter* spp.
Yeasty rot	*Saccharomyces* spp.	*Saccharomyces* spp.

[a]This is usually the more persistent state in which the fungus survives for long periods in the environment.
[b]This is the state in which the fungus usually occurs on the fruit.
Source: Data from References 42–44, 69, 83, 90, 102–105.

fungi, for example *Colletotrichum gloeosporioides*, which causes major spoilage of tropical fruits, are able to penetrate the intact cuticle of leaves, stems, and fruits. Following germination of the mold spore on the plant surface, a swelling (appressorium) is formed on the underside of the spore and from this appressorium a thin strand grows through the cuticle and into or between plant cells.[117] *Botrytis cinerea* can penetrate the flower parts, including petals, stigmas, styles, or stamens, of fruit such as strawberries, raspberries, and grapes. However, it may remain quiescent until conditions are favorable for it to resume growth and invade the fruit, often later in the growing season, during ripening or after harvest. The extent of preharvest fungal colonization is considered to be a primary determinant of postharvest decay of cherries.[33]

Other spoilage organisms enter the fruit mainly through mechanical injuries or through natural openings such as lenticels. Examples are *Penicillium expansum*, which is a major cause of spoilage of pome fruits and stone fruits, and *Erwinia carotovora*, an important cause of postharvest spoilage of field-grown tomatoes and of peppers. Spoilage of fruits is often initiated after harvest. Physical damage during harvesting and handling and exposure to stress conditions increase the susceptibility of the fruit. Consequently, microorganisms that have remained on the surface of the fruit are able to gain access to and attack the inner tissue. The ability of microorganisms to break down the tissue of fruits and to cause rots is associated with their ability to form cell wall–degrading enzymes, in particular, pectic enzymes.[22]

In many conditions the development of microorganisms in plant tissues is prevented by factors such as preformed inhibitors or inhibitors that are formed in response to injury or infection. Ripening is accompanied by weakening of cell walls and a decline in the ability of the fruit to produce antifungal substances. Susceptibility of the fruit to spoilage organisms generally increases and factors such as damage during harvesting and handling, and maintenance of the fruit in conditions of high humidity and high temperature, favor the development of spoilage.

27.5 MICROBIOLOGICAL SAFETY OF FRESH FRUITS

For many years travelers in various parts of the world have been aware of the risk of contracting disease as a result of the consumption of fresh fruits and other produce. Reports of the incidence of human pathogenic bacteria on fresh fruit have been summarized by Beuchat.[5] The proportion of outbreaks and cases of food poisoning associated with the consumption of fruits that are investigated and reported is probably low, but surveillance in the United States (US) indicates that the proportion of foodborne outbreaks that are associated with fresh produce has increased in recent years.[119] Factors that have contributed to this perceived increase may include improved reporting, larger and more centralized production and longer food chains, and an increase in global trade.[119] Examples of reported outbreaks of foodborne disease associated with contamination of fresh fruit are shown in Table 27-4.

Contamination of apples with manure from grazing cattle was probably the cause of outbreaks of infection with *Salmonella, Escherichia coli* O157, and *Cryptosporidium parvum* associated with unpasteurized apple juice. The use of oranges that have fallen to the ground, where they have been contaminated with *Salmonella,* and have then been processed without adequate washing was suspected as the cause of infection associated with unpasteurized orange juice (Table 27-4).[119] Contamination of raspberries before harvest may have contributed to an outbreak of infection with *Cyclospora* in 1996.[119] Pickers were suspected as the cause of contamination of raspberries in an outbreak of hepatitis A infection in Scotland in 1983.[92]

The pH of many fruits is lower than 4.0 and this low pH, combined with the presence of organic

Table 27-4 Examples of Reported Outbreaks of Foodborne Disease Associated with Contamination of Fresh Fruit

Outbreak	*Type of Fruit or Fruit Product*	*No. of Cases*	*No. of Deaths*	*Factors Suggested To Have Contributed to Outbreak*	*Reference*
1980, Canada **HUS**[a]	fresh-pressed apple cider	14	1	NR[b]	4, 114
***Escherichia coli* O157**					
1991, US	unpasteurized apple cider	23	0	Most of apples used were "drops."[c] Contamination may have occurred as a result of exposure to bovine or deer feces.	4
1996, US, Washington state and other states	unpasteurized apple juice	45	NR	NR	16
1996, US, Connecticut	unpasteurized apple cider	14	0	Some "drop" apples were used.	17
***Salmonella* spp.**					
1954, US, *S. miami*	sliced watermelon	17	1	Possible contamination with organism from surface when slicing. Possible injection of water into melons to increase weight.	40
1974, US, *S. typhimurium*	unpasteurized apple cider	NR	NR	Use of some "drop" apples from an orchard fertilized with manure.	12, 17
1979, US, *S. oranienburg*	precut watermelons	18	0	Damaged fruits were cut, covered with plastic film, displayed sometimes without refrigeration until sold.	9, 13, 14
1989–90, US, *S. chester*	cantaloupes from Mexico and Central America	>245 (estimated 25,000)	2	Not known. Cantaloupes were obtained from salad bars.	14, 118
1990, US, *S. javiana*	tomatoes	174	NR	Contamination of water bath used by a packer.	14, 119

continues

Table 27-4 continued

Outbreak	*Type of Fruit or Fruit Product*	*No. of Cases*	*No. of Deaths*	*Factors Suggested To Have Contributed to Outbreak*	*Reference*
1991, US, *S. poona*	fruit salads containing sliced cantaloupes from Texas and/or Mexico	>400	NR	Contamination from the surface of melons during slicing; possible multiplication on the sliced fruit.	14, 118
1993, US, *S. montevideo*	tomatoes	NR	NR	Contamination of water bath used by a packer.	15, 45
1995, US, *S. hartford*	unpasteurized orange juice	>32	NR	Contamination of fruit in orchard or during processing.	24
1998–1999, US, *S. typhi*	mamey, frozen, imported	~13	NR	Not known.	39
***Shigella* spp.**					
1998, UK, *S. flexneri*	fruit salad	36	0	Not known.	78
Cryptosporidium parvum					
1993, US	unpasteurized apple cider	>160	0	Use of "drop" apples from trees adjacent to an area grazed by cattle whose stool contained *Cryptosporidium.*	72
1996, US, New York state	unpasteurized apple cider	>20	0	Not known.	17
Cyclospora cayetanensis					
1996, US, Canada	raspberries imported from Guatemala	>1000	NR	Suggested as due to spraying fruit with insecticides and fungicides that were mixed with contaminated water.	48, 119
1997, US	raspberries, some imported from Guatemala	>140	NR	Not known.	19
Hepatitis A virus					
1983, UK	raspberries, frozen in UK	24	0	Suspected contamination by infected picker(s).	92
1990, US	strawberries, frozen, from California	28	0	Suspected contamination by infected picker(s).	77
1994, US	tomatoes, diced	92	NR	Suspected contamination by infected food handler.	132
1997, US	strawberries, frozen, imported	>258	NR	Possibly contaminated during harvesting.	18,49
Norwalk virus					
1987, UK	sliced melon	206	0	Infected food handler.	53
1990, Hawaii	fresh-cut fruit	>217	0	Infected food handler.	47
Calicivirus					
1998, Finland	raspberries, frozen, imported	~108	NR	Not known.	85

[a] HUS = hemolytic uremic syndrome.

[b] NR = not reported.

[c] "drops" = fruit picked from the ground.

acids, generally prevents the multiplication of foodborne pathogenic bacteria. Nevertheless, these bacteria may survive for a sufficient time to cause foodborne disease. The pH of cantaloupe and honeydew melons is in the region of 6.2–6.7 and that of watermelons is between 5.8 and 6.0. If these melons are sold as cut pieces any foodborne pathogens that are present may multiply on the cut surfaces of these and other fruits with a similar pH. *Salmonella* spp. and *Shigella* spp. have been shown to multiply on the cut surface of watermelons, cantaloupes, honeydew melons, papaya, and jacima fruits at 23° to 27°C.[34,40,41] *Salmonella* spp. have also been reported to multiply at 22°C on the cut surface of tomatoes with a pH between 3.99 and 4.37,[1] and *Salmonella montevideo* survived better at the stem scar and in growth cracks on tomato skin, and multiplied on puncture wounds and on tomato slices at 25°C.[129] *Escherichia coli* O157:H7 multiplied on cubes of cantaloupe and watermelon at 25°C and on the rind surface at 25°C and high humidity.[27] *Campylobacter jejuni* was reported to survive on sliced watermelon and papaya for sufficient time to present a risk to the consumer.[11] Initiation of bacterial soft rot may result in conditions that favor survival and growth of *Salmonella* in fruit.[130]

Strains of some species of mold including *Penicillium, Aspergillus,* and *Fusarium,* which may be present on fruits, can produce mycotoxins (Chapter 53), but because moldy fresh fruits are unlikely to be ingested, mycotoxins are of less importance in fresh fruits than they are in processed products such as fruit juices and dried fruits. The formation of patulin by *Penicillium expansum* in rots occurring in apples is a major problem in the production of apple juice (see Chapter 31), and the formation of aflatoxin is a problem in dried figs (see Section 27.9).

27.6 CONTROL OF MICROBIOLOGICAL SPOILAGE OF FRESH FRUITS

27.6.1 Preharvest Measures

Measures to prevent both field disease and postharvest spoilage start in the field. Cultivars differ greatly in their susceptibility to disease. Plant breeding and "genetic engineering" aim to produce new cultivars that are resistant to disease and have improved postharvest quality. For example, melons with a thick skin and raspberries with a firm texture are less susceptible to damage and therefore to infection.

The state of health of the planting material, whether seeds or cuttings, is an important factor in reducing disease and spoilage. Many fungi and bacteria persist in or on planting material, and can cause disease in the field and after harvest. Examples are the organisms that cause black rot of pineapples and bacterial canker of tomatoes. Plant health regulations exist to limit the movement of disease organisms within and between countries, and seed certification schemes have been established for many crops.

During growth, cultural practices can be used to reduce disease and spoilage.[62] Factors such as density of planting, orientation of rows, shading, mulching, tillage, irrigation, fertilizer regime, weed control, and application of fungicides all influence the health of the crop. Cultural practices such as pruning of fruit trees, destruction of crop debris, and crop rotation are important. Integrated pest management (IPM, in which "pest" encompasses "disease" and "weed") aims to coordinate control measures in the most efficient way.[93] This includes the use of fungicides and of biological control.

27.6.2 Harvesting and Handling Techniques

The choice of harvesting date and its relation to the maturity of the crop is of great significance, especially for fruits stored for extended periods.[35,61] Fruits destined for the fresh market are harvested mainly by hand; human pickers can select for maturity and, with proper training, can keep damage to a minimum. Machines harvest the entire crop at one "pass" and can cause substantial damage even when used correctly. Mechanical harvesting is used mainly for the more "robust" commodities and for crops that are intended for immediate processing. Further handling of the crop during transport and packing has a major effect on damage and infection. The presence of plant debris and cull-piles of rotting produce is a source of infection for the crop during harvesting and in the packhouse after harvesting.

27.6.3 Postharvest Treatments

Many chemicals, including fungicides, bactericides, and plant growth regulators, are applied to fruits after harvest.[123] Regulations for approval of the use of these chemicals are designed so that residues in or on the fruit should be less than the maximum residue levels. These are specified under European Community Directives that are implemented in Pesticides Regulations in the UK,[113] maximum residue levels are specified in the US by the Environmental Protection Agency and in other countries by similar organizations. Imported fruits must comply with the maximum residue levels of pesticides specified in the importing country. Postharvest use of chemicals and the resulting residues have been reviewed by Papadopoulou-Mourkidou.[80] Because of the risk of development of fungicide-resistant strains of fungi, it is recommended that a range of chemically unrelated compounds be used at different stages, for example, before and after harvest and before and after storage for repacking. As examples of use, sodium orthophenylphenate (SOPP) and either thiabendazole or imazalil are used to prevent spoilage of citrus fruit by *Penicillium* spp.; thiabendazole or imazalil are used to prevent postharvest wastage of bananas; and a mixture of carbendazim and metalaxyl is used for the treatment of apples, and iprodione for pears. Thiabendazole is active against a wide range of fungi. Imazalil, prochloraz, and iprodione are effective against alternaria rot. Etaconazole, propiconazole, and guazatine were among the first compounds effective against geotrichum sour rot. Dichloran is effective specifically against rhizopus rot, and metalaxyl and fosetyl aluminium inhibit phytophthora rot. There is an increasing interest in biological control of postharvest diseases.[20,32,55,68,134]

Heat treatment of fruit using hot water at 40° to 60°C for between 30 seconds and 10 minutes, or hot air at 43° to 54°C for 10 to 60 minutes, or hot water containing fungicides may be useful in reducing fungal spoilage of some fruits.[2]

The main antibacterial in the postharvest treatment of fruits is chlorine used in washing water, for example, for tomatoes and peppers. Care is needed to maintain an effective concentration of free, available chlorine, and the effect of the chlorine is mainly to prevent the spread of bacteria in the wash water rather than to kill bacteria on the surface of the fruit. Chlorine dioxide is used also to prevent the spread of bacteria in wash water.

Growth-regulating chemicals are used widely after harvest; ethylene is used to hasten ripening, and auxins and gibberellins to retard ripening. Some fruits, particularly apples, are treated with a calcium drench, which helps to protect against physiological disorders and fungal decay.[23,56] Semi-permeable coatings can reduce respiration rate.[26] Artificial waxes inhibit moisture loss, may also modify respiration, enhance the appearance of some commodities, and may reduce spoilage. Waxes may also be used as a vehicle for a postharvest fungicide, eg, for the application of imazalil to citrus fruits. Fruits such as citrus may be enclosed in individual plastic film bags, which are then heat-shrunk ("seal-packaging"). This, in conjunction with fungicide treatment, can extend storage life.[101] These treatments to optimize the physiological condition of the fruit also minimize its susceptibility to microbial spoilage.

Irradiation has potential uses in the preservation of fruits. In most cases, fruits are more sensitive to irradiation than are microorganisms, an exception is the strawberry. Thus the major aims of irradiation of fruits are to delay ripening and for disinfestation.[131] Combinations of irradiation and other treatments have been investigated for reduction of the microbial load on fruits.

27.6.4 Cooling

Cooling promptly after harvest is important to remove field heat and to lower the temperature of the fruit near to its optimum storage temperature.[120,122] It is recommended that highly perishable temperate fruits such as strawberries and apricots be cooled to as low a temperature as possible, above 0°C, within 6 hours of harvest.[59] The majority of other fruits should be cooled to their optimum storage temperature within 12 hours of harvest, with the exception of melons and certain stone fruits. Hydrocooling by spraying, drenching, or immersion is used for fruits such as apples, peaches, and melons, although cooling with cold air is used more commonly. Hydrocooling by immersion has the disadvantage that cooling water and contaminating microorganisms may be drawn into the fruit through the stem scar, stomates, or damaged tissue as it cools.[3,136]

27.6.5 Storage

Storage at optimum temperature and relative humidity is the most important method of maintaining the quality of fresh fruits and minimizing spoilage during storage.[42,60,74,120] Storage in optimum conditions maintains the quality of fruit by minimizing loss of water, slowing the rate of respiration, minimizing susceptibility to microbiological spoilage, and inhibiting the growth of microorganisms. Some fruits can be stored at temperatures near 0°C, which severely limits the rate of growth of microorganisms, but others, especially tropical and subtropical fruits, suffer physiological damage (chilling injury) if maintained at relatively low temperatures (in some cases as high as 14° to 16°C),[127] making the injured tissue especially prone to fungal invasion, particularly by *Alternaria* and *Cladosporium* spp.

Controlled atmospheres are used for some fruits,[121] notably for apples, pears, and avocados. In some cases, controlled atmospheres containing optimum concentrations of oxygen and carbon dioxide may slow the respiration rate and ripening changes. Removal of ethylene generated by the fruit helps to delay the onset of ripening. Examples of the optimum conditions for storage of selected fruits are shown in Table 27-5.

27.6.6 Packaging

Prepacks of produce for retail sale are sometimes prepared before shipment but more usually are prepared shortly before distribution to retail outlets. Sealed plastic films, with appropriate permeabilities to gases and to moisture, can be used to form packs in which the atmosphere is modified partly by packing under an appropriate gas mixture and partly by the respiration of the fruit.[61,95] The modifed atmosphere is liable to alter during the shelf life of the produce but it retards deterioration of the produce in a similar way to controlled atmosphere. Modified atmosphere packaging is used, for example, to extend the shelf life of apples and tomatoes.

27.7 CONTROL OF MICROBIOLOGICAL SAFETY OF FRESH FRUITS

27.7.1 General Principles

Contamination of fruit with foodborne pathogenic microorganisms can occur at all stages of growth, harvesting, handling, and marketing. Conditions in the field during growth and harvesting are a major critical control point[51] and contamination at this point is difficult to eliminate later by treatment with antimicrobial agents. In a guide to minimizing microbial food safety hazards for fresh fruit and vegetables, the US Food and Drug Administration (FDA)[38] stressed the following principles:

1. Prevention of microbial contamination of fresh produce is favored over reliance on corrective actions once contamination has occurred.
2. To minimize microbial food safety hazards in fresh produce, growers, packers, and shippers should use good agricultural and management practices in those areas over which they have control.
3. Fresh produce can become microbiologically contaminated at any point along the farm-to-table food chain. The major source of microbial contamination with fresh produce is associated with human or animal feces.
4. Whenever water comes in contact with produce, the source and quality of the water dictate the potential for contamination. The potential for microbial contamination from water used with fresh fruits and vegetables should be minimized.
5. Practices using animal manure or municipal biosolid wastes should be managed closely to minimize the potential for microbial contamination of fresh produce.

Table 27-5 Examples of Recommended Conditions for Commercial Holding and Storage of Selected Fruits[a]

Fruit	*Temperature Range (°C)*	*O_2 Concentration (%)*	*CO_2 Concentration (%)*
Apples	0–4	2–3	1–2
Apricots	−1–0	2–3	1–2
Grapes	−1–0.5	2–5	1–3
Peaches	−1–0	1–2	5
Pears	−1–0.5	2–3	0–1
Plums	0–1	1–2	0–5
Strawberries	0	10	15–20
Avocados	5–13	2–5	3–10
Oranges	2–8	5–10	0–5
Grapefruits	10–15	3–10	5–10
Lemons/Limes	10–14	5–10	0–10
Pineapples	7–13	5	10
Bananas	13–15	2–5	2–5
Tomatoes, mature green	12–15	3–5	—
part-ripe/ripe	10–12	3–5	—
ripe	8–10	—	—

[a]Many of these fruits are usually stored in refrigerated air rather than in controlled atmospheres.

Source: Data from Kader[58] and Snowdon.[103,104]

6. Worker hygiene and sanitation practices during production, harvesting, sorting, packing, and transport play a critical role in minimizing the potential for microbial contamination of fresh produce.
7. All applicable local, state, and federal laws and regulations for agricultural practices, or similar laws, regulations, or standards for operators outside the US, should be followed.
8. Accountability at all levels of the agricultural environment (farm, packing facility, distribution center, and transport operation) is important to a successful food safety program. There must be qualified personnel and effective monitoring to ensure that all elements of the program function correctly and to help track produce back through the distribution channels to the producer.

Evidence shows that several of the outbreaks shown in Table 27-4 associated with *E. coli* O157, *Salmonella*, and *Cryptosporidium* have resulted from contact of fruit with animal manure. Untreated, improperly treated, or recontaminated manure or biosolids may contain pathogens that can contaminate produce.[38] Some microorganisms, including vegetative bacteria, can survive for long periods after application to soil.[51] Composting of manure can reduce the number of pathogenic microorganisms present but adequate composting treatment is needed in order to ensure that all the compost reaches a sufficiently high temperature for sufficient time to ensure maximum reduction of pathogenic microorganisms. Several guides to composting treatment have been published in the US.[38] Treatment of sewage reduces the level of microorganisms but the numbers that remain can cause contamination of crops. The use of treated sewage sludge as fertilizer must also be controlled closely to minimize the survival and spread of pathogenic microorganisms. In order to avoid a source of contamination of fruits, such as apples and pears, fruit should not be collected from the ground in orchards used for grazing.

Some of the outbreaks shown in Table 27-4 were attributed to contamination of water applied to crops. Measures are needed to minimize contamination of surface water and ground water with pathogenic microorganisms and to prevent contamination of crops from water supplies in the field during irrigation, harvesting, and processing.[38]

Some outbreaks may have been caused by contamination of crops by workers in the field. Others have resulted from contamination during processing and retail sale. In order to prevent this, detailed guidance for good hygienic practices by workers during growing, packing, holding, and transport of food is available.[38]

Several outbreaks of salmonellosis have been associated with melons (Table 27-4). Because melons are grown on the ground, it may be difficult to prevent contamination with foodborne pathogenic microorganisms. Sampling of melons imported into the US in 1990 and 1991 showed that many serotypes of *Salmonella* were isolated from approximately 1% of rinds.[14] While large companies were reported to wash melons and dip them in chlorine solutions, field-packed melons did not receive such treatment.[118] If melons with a contaminated rind are cut, the edible part may be contaminated by the cutting knife and maintenance of the cut melon at ambient temperature can result in growth of pathogenic bacteria. It is recommended, therefore, that melons be cleaned thoroughly with potable water before cutting, that clean and sanitized utensils and surfaces be used, and that cut melons be kept at a temperature lower than 7°C until served or sold.[14] If not refrigerated, cut melons should be kept for less than 4 hours. In addition, transfer of *S. montevideo* into the inner tissue of tomato by cutting has been demonstrated.[64]

27.7.2 Treatment of Fruits with Antimicrobial Agents to Inactivate Pathogenic Microorganisms

Prevention of contamination is preferred over corrective action once contamination has occurred.[6,38] Antimicrobial chemicals such as chlorine are useful in preventing the accumulation of microorganisms in processing water. These chemicals are less effective in inactivation of microorganisms on produce but, if properly controlled, can reduce numbers. If fruit is treated by immersion in wash water containing an antimicrobial agent, the temperature of the wash water should, ideally, be at least 10 degrees C higher than that of the fruit, in order to minimize uptake of water and of contaminating microorganisms through stem tissue, stomates, or damaged tissue.[6] Washing with water can reduce the number of microorganisms on produce but even with the addition of the usual concentrations of antimicrobials (eg, 100 mg free available chlorine l^{-1} at pH 6.5 for 20 minutes[67]), the reduction may only be of the order of 10- to 100-fold.[6,38]

Chlorine, added as a gas or as hypochlorite, is hydrated in solution to form hypochlorous acid, which has a pK of about 7.5 and dissociates to give the hypochlorite ion. The antibacterial activity of chlorine solutions is due mainly to hypochlorous acid and is influenced strongly, therefore, by the pH of the solution. A pH of 6.5 to 7.0 is suitable; below pH 6.0 hypochlorite solutions are too unstable for use. Because chlorine combines rapidly with organic materials and is then largely inactivated, it must be added repeatedly to maintain the required concentration of free, available chlorine (chlorine present as hypochlorous acid plus hypochlorite). The upper limit on the total concentration of chlorine used will depend on any damaging effect on the plant tissue and on the occupational exposure limit for workers.[6] When tomatoes and apples (to which wax had been applied before purchase) inoculated with *Salmonella*, *E. coli* O157, or *Listeria monocytogenes* were treated by spraying with a solution containing 2,000 ppm chlorine in 0.05 M phosphate buffer pH 6.8, the number of these pathogens was reduced by between 0.35 and 2.30 log cfu cm^{-3} more than by similar treatment with water.[8]

In addition to chlorine, treatments with chlorine dioxide, trisodium phosphate, organic acids, hydrogen peroxide, and ozone have been investigated as a means of decontamination of produce. In the US, chlorine dioxide at a maximum concentration of 200 ppm is allowed for treatment of equipment for processing fruit and vegetables, but a maximum concentration of 5 ppm is allowed in water for washing whole, fresh fruit.[6] Concentrations of up to 5 ppm can be useful for reducing the numbers of microorganisms in wash water but are not effective in reducing numbers on the surface of fruit. Ozone may be useful for preventing a build-up of microorganisms in process water. None of the alternative antimicrobial chemicals appears to be more effective than chlorine for decontamination of fruits. Irradiation might be a means of reducing vegetative bacterial pathogens on raw fruits but only if this treatment proved to be economic and acceptable to consumers[6] and if it is effective in conditions that do not damage the fruit.

27.8 FROZEN FRUITS

27.8.1 Processing and Its Effect on Microorganisms

Some fruits, such as strawberries, raspberries, and cherries, may be frozen whole. These and other fruits are sometimes sliced before freezing or may be frozen as sweetened products blended with sugar.[91] They are also chopped and sieved and frozen as purees. Fruits such as cherries are pitted before freezing and may be blanched in order to inhibit enzymic activity.[70] Slicing and sieving will tend to introduce microorganisms from the processing equipment and the addition of sugar is likely to protect microorganisms from damage during freezing. Freezing and storage at freezing temperatures can damage microorganisms, and freezing in acid conditions is liable to damage food poisoning bacteria more than freezing at neutral pH (see Chapter 6). The outbreaks of infection with *Salmonella typhi* and with viruses transmitted on frozen fruit (Table 27-4) illustrate the ability of these microorganisms to survive the freezing process. Growth of microorganisms is prevented in frozen foods but microorganisms are liable to survive freezing (and thawing), and they may die only slowly during storage of the frozen fruit (see Chapter 6).

27.8.2 Control of Microbiological Safety and Quality of Frozen Fruits

Evidence from the few reported outbreaks of disease associated with frozen fruits and information about the effect of freezing on microorganisms (see Chapter 6) indicate that major factors in controlling the microbiological safety and quality of these products are limitation of the microbiological contamination of the fruit used and the use of good hygienic practices during processing (Section 27.7.1).

27.9 DRIED FRUITS

27.9.1 Processing and Its Effect on Microorganisms

Fruits that are dried commonly include raisins, prunes, dates, apples, figs, apricots, and peaches. Many are sun dried, giving a product with less than 25% water.[109] Dehydrated fruits, which are often used in other products, are produced by a process that reduces the water content to 1% to 5%. Because the water activity (a_w) of most sun-dried fruits is less than 0.65, they do not support the growth of microorganisms. Some types of fruit, including peaches, apricots, pears, and bananas, are treated with 1,000–4,000 mg sulfur dioxide (SO_2) l^{-1} before drying, to inhibit enzymic browning; this treatment also destroys many of the microorganisms that were on the original fruit. Other fruits, including prunes, dates, figs, and vine fruits, are not necessarily treated with sulfur dioxide.[52] Some of the factors that are important in relation to the microbiology of dried fruits are illustrated by consideration of prunes, raisins, and figs.

Prunes

A prune is defined as a dried plum with a moisture content reduced to 19% to 35%,[106] a_w 0.65 to 0.83.[10] The major sources of prunes are the US (California), France, and Chile.[106] Production in California was described by Somogyi.[106] Prune plums are allowed to ripen on the tree. Most of the crop is shaken from the tree by mechanical shakers, caught on canvas frames, transferred to bins, and transported for dehydration (this has replaced sun drying). Before drying, the fruits are cleaned with air blast and water sprays and dipped in water. They are then spread on trays and dried, usually under forced-air flow for about 24 to 36 hours, to about 18% moisture, then sorted and packed. For prunes with a moisture content higher than 25% or a water activity above 0.7 either pasteurization or the addition of potassium sorbate, as a dip or spray, is recommended. In order to improve ease of handling for some purposes, coatings are applied. These coatings may be vegetable oils, mono- and di-glycerides, dextrose, starch, rice powder, or, for prunes with <4% moisture, calcium stearate. In order to supply a ready-to-eat product, dried fruits may be rehydrated to a water activity of 0.85 to 0.90 before packing; they are treated with hot or boiling water, which destroys many of the contaminating microorganisms. Up to 0.1% potassium sorbate or sodium benzoate is added to these high-moisture dried prunes to prevent growth of yeasts and molds.

Raisins

Drying of grapes to produce raisins is described by Striegler, Berg, and Morris.[115] The major producing countries between 1979 and 1993 were Turkey and the US.[37,115] For the production of raisins in California, grapes may be sun dried on trays in the field for more than 7 days and then cleaned on a screen shaker to remove foreign and substandard material. Depending on weather conditions during sun drying, mold growth tends to occur on the grapes. Reconditioning is used to improve the quality of such raisins. This process involves passage over screen shakers, washing in hot water (70°–80°C), and further drying on trays at about 60°C for 20 to 25 hours.

Raisins are also produced by dehydration in bulk bins. They are first dipped in hot water (80°C + 0.25% NaOH or 99°C), washed in fresh water, and spread on drying trays. Golden seedless raisin grapes are then exposed to SO_2 gas at 2,000 to 4,000 mg l^{-1} for 4 to 6 hours, whereas dipped seedless raisin grapes are not treated with SO_2. Both types are then dried in a counterflow of hot air at temperatures in the range 57° to 74°C for about 24 to 36 hours.

In the past, unprocessed raisins were stored commercially in stacks and fumigated periodically with chemicals such as methyl bromide or phosphine to eliminate insect infestation. Because of environmental concerns, alternative methods are now being used, in particular storage in stacks that are purged of oxygen and in which a low-oxygen atmosphere is maintained long enough to kill any insect pests.

During processing, raisins are treated further to remove extraneous material, to break up clusters into individual raisins, and to grade into sizes. They are then spray washed with cold water, passed through wash tanks, passed over a dewatering shaker and through further cleaners, inspected, and sorted. A coating of food-grade oil or other material may be applied depending on the requirement of the end user. In the US, the final product is inspected by US Department of Agriculture (USDA) inspectors and must have a moisture content of <18%.

Figs

Figs have an unusual development and structure. The small flowers are enclosed in a hollow fleshy structure derived from stalk tissue, which has a small opening, the ostiole.[29,31,103] Pollination of most cultivars is by a tiny wasp, which passes through the ostiole. Other insects and fungi can also enter through the ostiole and develop in the interior of the cavity. Some fig cultivars are self-fertile, but these also have an open ostiole through which insects and fungi can enter the cavity. *Fusarium moniliforme,* which causes soft rot of figs, is transmitted into the fig by the fig wasp, and yeasts and bacteria are carried into the fig by fruit flies.

Figs are usually sun dried, often on the ground after falling, and growth of fungi may occur during the drying process. Growth of *Aspergillus* spp. may occur giving rise to formation of aflatoxin in figs.

Glacé Fruits

Glacé fruits are produced by infusing blanched fruit with increasingly concentrated glucose syrups that contain SO_2 as a preservative.[52] The product is then dehydrated at low temperature to reduce the a_w to about 0.85. This processing destroys the majority of the initial microflora.

27.9.2 Control of Microbiological Spoilage and Safety of Dried Fruits

As with other fruit products, practices that limit microbiological contamination in the field, rapid drying, and reduction of contamination on equipment used in transport and processing can help to lower the microbial load on the product. Treatment with sulfur dioxide destroys many of the microorganisms on the surface of fruits. Dried fruits with an a_w lower than 0.65 are not susceptible to microbial spoilage. Unsulfured dried fruits with an a_w higher than 0.65 are liable to spoilage, particularly by *Eurotium* species (see Chapter 7), and potassium sorbate may be added as a preservative. High-moisture prunes are susceptible to spoilage by *Eurotium* species and *Xeromyces bisporus* unless they are treated with an antifungal compound or heated in a hermetically sealed container (see Chapter 7). Potassium sorbate is added commonly to high-moisture dried fruits. Growth of yeasts and molds on high-moisture prunes was limited by the addition of up to 0.1% potassium sorbate or sodium benzoate.[98]

Several surveys have shown unacceptable levels of aflatoxin in dried figs.[52] In the United Kingdom (UK) (and also in the European Community) the statutory limits for aflatoxin in dried figs are as follows:[73]

- 4 μg kg^{-1} total aflatoxin for dried figs and their products for sale or for incorporation in any compound food or for import for direct human consumption
- 10 μg kg^{-1} for such imports intended for further processing before sale or incorporation in any compound foods for human consumption.

Surveys between 1988 and 1992 of whole dried figs imported into the UK, mainly from Turkey, showed that during this period the percentage of figs containing >4 μg aflatoxin kg^{-1} decreased from 26% to 16%; however, some samples contained >50 μg kg^{-1}.[73] Consignments of whole dried figs and fig paste that contained >10 μg aflatoxin kg^{-1} were refused entry into the UK by port authorities. During the period of this survey, screening of figs under UV light was introduced, based on the correlation between blue/green/yellow fluorescence on the outside of figs and high aflatoxin level.[100] This technique offers a method for improved testing of samples.

Although SO_2 is used in the production of glacé fruits, it is only partially effective as an antimicrobial because free SO_2 is bound rapidly by glucose.[52] Spoilage of partially prepared glace pineapple by *Schizosaccharomyces pombe* has been described and associated with the resistance of this yeast to SO_2 combined with its ability to grow at reduced a_w.[52]

27.10 CANNED FRUITS

27.10.1 Processing and Its Effect on Microorganisms

In foods with a pH of 4.6 or lower, growth of *Clostridium botulinum* is inhibited (see Chapter 41). The thermal processes for acid canned foods, ie, foods with a pH of 4.6 or lower (US) or with a pH of 4.5 or lower (UK), are much less than an F_0 3 process required for low-acid foods (see Chapters 3 and 41) and are based on the need to inactivate vegetative microorganisms and spores that are able to result in growth in the final product. In the case of canned fruits, these treatments normally involve heating at <100°C. Following washing, peeling (with the use of alkali in peeling some fruits[89]), and grading, many fruits are blanched by heating briefly at 88° to 99°C to inactivate certain enzymes that would cause deterioration of flavor and texture. The fruits may then be exposed to sulfur dioxide either by dipping in a solution containing 2,000 to 4,000 mg SO_2 l^{-1} for 2 to 5 minutes or by fumigation. Acidulants and antioxidants such as ascorbic acid are often added, and sugar may be included. If heat treatment during canning is insufficient, canned fruits are liable to spoilage by surviving microorganisms.

Canned Fruits with a pH in the Range 3.7 to 4.6

These products include canned fruits such as tomatoes, pears, and figs. There are reports of spoilage by spore-forming bacteria including the butyric acid clostridia, *Clostridium pasteurianum* and *C. butyricum*, and also by *Bacillus coagulans*. Spores of *B. coagulans* are reported to be more heat resistant than those of *C. pasteurianum* and *C. butyricum* but spoilage by the *Bacillus* appears to require a higher pH than spoilage by the *Clostridium* spp.

Clostridium pasteurianum or similar bacteria that were reported as the cause of canned fruit spoilage could initiate growth from vegetative bacteria in pear and apricot juices with a pH of 3.6.[54,123] Bacteria similar to *C. pasteurianum* were reported as the cause of spoilage and swelling of batches of canned pineapple with a pH of 4.4.[107,108] *C. pasteurianum* and *C. butyricum* have been reported to cause spoilage of canned tomatoes.[46] Organisms similar to *C. pasteurianum* were reported to multiply and cause spoilage in canned mandarin orange at pH 3.90 to 3.97 and higher, and consequently it was recommended that the product should be adjusted to a pH below 3.5.[50] Commercially canned pears were reported to have a pH in the range 4.1–4.3 and a water activity (a_w) of about 0.985;[54] two strains (both *C. pasteurianum*) of seven strains of butyric anaerobes tested multiplied in pear juice in these conditions. Adjustment of the water activity of pear juice with sugars was suggested as a means of preventing growth of *C. pasteurianum*, and it was concluded that for an a_w range of 0.97 to 0.98 the pH of the product should be less than 4.0. It was suggested that appropriate combinations of pH and a_w might reduce the heat processing needed for canned pears.

Some acid and acidified canned fruits, in particular tomato products, are susceptible to spoilage by *Bacillus coagulans* resulting in "flat-sour" spoilage, so called because the bacteria produce acid but no gas from carbohydrates, cause off-flavors, and do not cause swelling of cans. In the mid-1950s, it was realized that the pH of some tomatoes was in the range 4.5–4.8.[87] At pH higher than 4.3, the risk of spoilage of canned tomatoes increases, and in the range pH 4.5–4.8, the risk of botulism appears. Therefore, in canning high-pH tomatoes, organic acids should be added to lower the pH.[21,87] The vegetative cells of some strains of *B. coagulans* can multiply in culture media at pH 4.05 and in tomato juice at pH 4.15 to 4.25, but heated spores failed to germinate and give growth in tomato juice adjusted to a pH lower than 4.3.[36,82] *Bacillus coagulans* is described as a thermophilic or facultatively mesophilic bacterium[116] and the temperature range for its growth was reported as 28° to 65°C,[36] but growth at temperatures as low as 18°C has been reported.[99] This bacterium originates from soil, it can become established in tomato processing plants, and large numbers may accumulate in water used for washing tomatoes. Because sugar and starch may be sources of the bacterium, products used in canned fruit should be subject to standards for these bacteria.

The recommended sterilization value of a process for whole canned tomatoes in juice against spores of butyric acid anaerobes when the pH is above 4.3 was the equivalent of 10 minutes at 200°F (about 93°C) and assuming a z-value of 15 degrees F (8.3 degrees C) (see Chapter 3 for a definition of z value), and when the pH is less than 4.3, the equivalent of 5 minutes at 200°C.[76,135] Against spores of *B. coagulans* at pH 4.5, the recommended process was the equivalent of 0.7 minutes at 250°F (121°C) assuming a z-value of 18 degrees F (10 degrees C). York et al.[135] concluded that a marginal heat treatment of tomato products containing spores of *B. coagulans* so damaged the spores that they were unable to germinate and give growth in the tomato product at pH ≤4.3. They reported that at pH ≤4.3 after a heat treatment (calculated on the basis of the heating throughout the product) equivalent to heating at 212°F (100°C) for 1.56 minutes, assuming a z-value of 27 degrees F (15 degrees C), no organoleptic, or microbiological spoilage by lactic acid bacteria, butyric anaerobes, or flat sour bacteria was experienced either commercially or in the laboratory.

Outbreaks of gaseous spoilage of commercially canned fruits by bacteria of the *B. macerans/B. polymyxa* group have been reported.[126] The heat resistance of spores of these bacteria was reported to be comparable with that of the butyric anaerobes. In phosphate buffer pH 7.0, heating at 100°C for about 30 minutes was required to inactivate 10^5 spores of a strain of *B. macerans,* whereas in canned fruit syrup, pH 3.6, heating at 100°C for 1 minute was required. The spoilage was attributed to post-pasteurization contamination of cans by entry of cooling water; the bacteria were present in cooling water and the spores would be sufficiently heat resistant to withstand the temperatures in rotary coolers.[126]

Canned Fruits with a pH Lower than 3.7 to 4.0 (High Acid)

These products are subject to spoilage mainly by molds and to a lesser extent by yeasts and lactic acid bacteria. The ascospores of certain molds, in particular ascospores of *Byssochlamys, Neosartorya, Talaromyces,* and *Eupenicillium* species,[84,109] have the potential to survive the heat treatments used commercially in canning acidic fruits. The main organisms causing spoilage of acidic canned fruits appear to be *Byssochlamys fulva* and *B. nivea.* The following *D* values have been reported for ascospores: *B. fulva* and *B. nivea* in grape juice at 86° to 87.8°C, between 4.8 and 11.3 minutes[7]; *Talaromyces flavus* in apple juice at 92°C, 2.2 minutes; *Neosartorya fischeri* in apple juice at 85°C, 18 minutes; *Eurotium herbariorum* in grape juice at 70°C, 2.5 minutes.[109] *Byssochlamys* spp. are obligate aerobes, but they can grow in very low concentrations of oxygen.[84] Even limited growth may be sufficient to allow the production of pectin-degrading enzymes that cause softening and ultimately complete breakdown of the fruit. Mycotoxin formation by molds of all these genera growing in fruit products has been reported. Some strains of *Byssochlamys* are reported to form byssochlamic acid, patulin, byssotoxin, and malformins[7,109]; production of very low levels of patulin by *Byssochlamys* species in canned fruits has been reported. Fields and orchards are the source of the fungus, which probably survives in plant debris. In New York, *B. fulva* was the predominant mold on sound and decayed grapes, grape vegetation, apples, blackberries, cherries, peaches, raspberries, apricots, pears, and orchard and vineyard soils.[7]

Spore-forming bacteria with a pH optimum at pH 3.4 to 4.0 have been isolated from spoiled fruit juice beverages[111] and subsequently identified as *Alicyclobacillus.*[86,112] Spores of one strain heated in apple juice, pH 3.5, had a D value at 95°C of 2.8 ± 0.7 minutes and a z-value of 7.7 degrees C. Spores of a second strain heated in grape juice, pH 3.3, had a D value at 95°C of 2.4 ± 0.7 minutes and a z-value of 7.2 degrees C, indicating that they could survive the thermal process given to many fruit juices and other fruit products. This organism is thermophilic growing at temperatures from 12° to over 53°C.[86] Spoilage of fruit juices by this bacterium has been reported rarely; it has been isolated from canned diced tomatoes[128] and its significance in relation to canned fruits remains to be determined.

There is a risk that microbial spoilage of some canned fruits may cause a rise in pH of the product and allow growth and toxin production by *Clostridium botulinum*. Between 1899 and 1975, 35 cases of botulism in the US were attributed to "acid foods," of which 34 were home processed and one was commercially processed.[79] In several of these incidents other microorganisms, in addition to *C. botulinum*, were isolated from the toxic product. There is evidence that, in certain conditions, prior growth of a yeast or mold or of *Bacillus licheniformis*, which has survived the heat treatment or has contaminated the product after heating, can raise the pH of the food allowing growth of *C. botulinum*.[75,79]

27.10.2 Control of Microbiological Spoilage and Safety of Canned Fruits

Soil is a source of spore-forming bacteria and there are several examples of situations in which spoilage of canned fruit has been associated with an unusually high level of soiling of the fresh fruit. Heat processes for canned fruit were designed to inactivate spoilage microorganisms at the concentration expected in these foods in conditions of good hygienic practice. The risk that heat-resistant microorganisms will survive heat processing can be reduced by using good quality fruit that shows no signs of latent infection, by minimizing contamination of the fruit with soil, by thorough washing, and by preventing build-up of microorganisms in wash water and on processing equipment. Because of the importance of minor ingredients as a source of contamination, particularly with spores of *Clostridium* and *Bacillus* spp., it is recommended that ingredients such as sugar, starch, and spices, used in canning should comply with standards limiting the number of spores of these bacteria present (see Chapter 35). Control of the pH for all canned foods is essential, and at the higher limit of the pH range, control of water activity may also be important. Prevention of leakage of cans during cooling and minimizing contamination of cooling water are also important (see Chapter 3).

27.11 CONCLUDING REMARKS

Throughout this chapter it is clear that conditions during growth of fruits influence contamination and affect the microbiological safety and quality of both fresh and processed products. Changes taking place in agricultural practices, including the greater use of animal waste and municipal waste on land; the increasing use of fruits grown in, and transported from, all parts of the world; the use of new techniques for processing including new methods of thermal treatment; and the development of novel types of product may give rise to new problems.

A significant proportion of fruit is liable to be wasted by spoilage after harvest. Much of this spoilage could be prevented by good agricultural practices that minimize the inoculum level of spoilage microorganisms, by the use of optimal storage conditions, and by careful handling so as to minimize physical damage to the produce. There is a continuing need to ensure that people handling fruit during harvesting, packing, transport, and retail sale do so in a way that minimizes damage to the product. Extreme care in the field and throughout postharvest handling can be wasted by careless handling during transport, display, and retailing.

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28 Cereals and Cereal Products

J. David Legan

CHAPTER CONTENTS

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28.1 INTRODUCTION—THE IMPORTANCE OF CEREALS

Cereals, the fruits of cultivated grasses of the monocotyledonous family Gramineae, are arguably the most important agricultural crops in the world. The eight principal cereal crops are wheat, rice, maize, barley, sorghum, millet, oats, and rye. Kent and Evers[73] explained the technology of cereals in some detail, including summary statistics for production and use.

The total world production of cereals has increased dramatically during recent history. In 1960, the total world cereal crop was over 900 million metric tons, whereas by 1987 to 1989 the annual crop had risen to 1,784 million tons. Wheat, rice, and maize were the dominant crops throughout this period and the majority of the total increase in production was due to larger crops of these three cereals. In 1960, their combined total was approximately 700 million tons, rising to about 1,500 million metric tons in 1989. Each provided a similar contribution to the total world harvest throughout this period but wheat has been slightly ahead of the other two since the early 1970s (Figure 28-1). The total world barley crop also doubled over the same period but was only 170 million metric tons in 1989. Production of the other four cereals—sorghum, millet, oats, and rye—hardly changed over the period. The increase in production has been associated with only a small increase in the total land area given over to cereals and is mainly due to higher yields.

Wheat is grown worldwide but is most successful in temperate to subtropical climates with moderate annual rainfall (229–762 mm) and a mean summer temperature of 13°C or more. Where winters are not too severe, seed can be sown in autumn. In countries with severe winters, wheat is sown as early as possible in the spring to be harvested before the first autumn frost. Two-thirds of the world wheat crop is used for human consumption and about a fifth for feedstuffs. About 7% is used for seed with the remaining small amount being used in industrial processing, such as starch production, gluten extraction, pharmaceuticals manufacture, and some waste.

Rice is grown in the tropics and some temperate regions. Although normally grown in water-covered fields, it can be grown on dry land. There are varieties that are adapted to a wide range of environmental conditions. Nearly all the world rice crop is used for human consumption.

Maize can be grown in tropical or temperate regions which have at least 90-day periods without frosts and gives its highest yields when it can mature in 130 to 140 days. A little under two-thirds of world maize production is used for animal feeds and only one-fifth for human consumption, although it is a

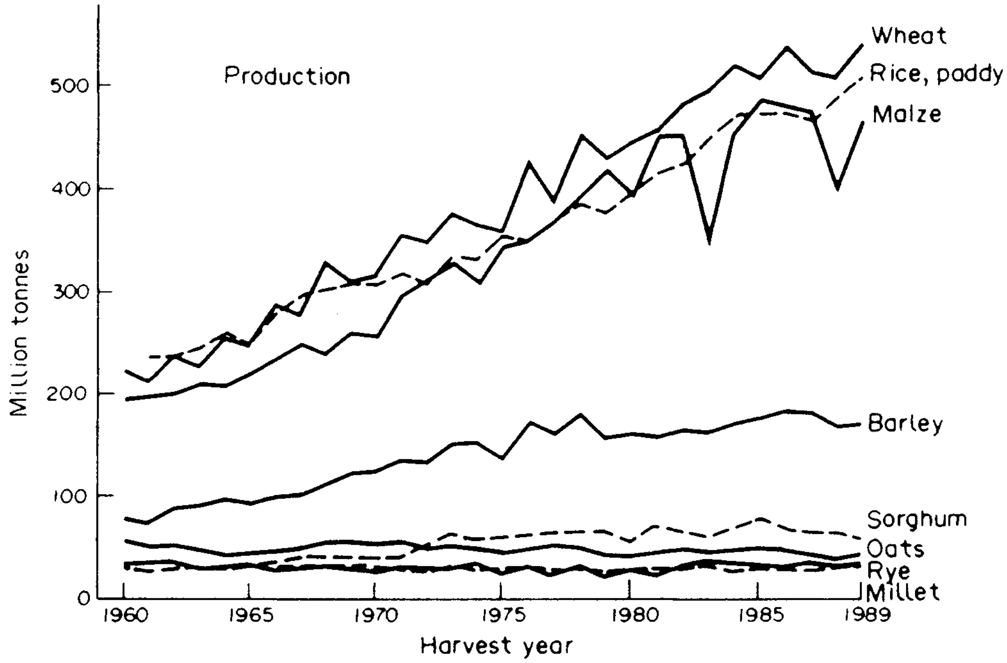

Figure 28-1 World Cereal Production 1960–1989. *Source:* Reprinted with permission from N.L. Kent and A.D. Evers, *Kent's Technology of Cereals,* 4th ed., p. 2, © 1994, Butterworth-Heinemann.

staple in some countries in Latin America and in parts of Africa. The remainder goes for industrial processing into starch, sugars and syrups, industrial spirits and whiskey, and about 1.5% for seed.

Barley is a temperate crop with a distribution and requirements similar to those of wheat. Its principal uses are in animal feeds, malting, brewing, and distilling.

Sorghum is grown in tropical regions (latitudes below 45°) and is favored by a mean temperature of 27°C. It forms the staple food in many parts of Africa, Asia, and Central America with one-third of the world crop being used for human consumption. Over half the total crop is used for animal feed and the remainder is used in industrial processing and as seed.

Millet is the name given to at least 12 species of small-seeded grasses. Often grown under adverse conditions of heat, limited rainfall, and unfertile soil, it is a relatively low-yielding crop but does better than other cereals in these conditions. Most millet is used in the country of production and three-quarters goes for human food. About 3% is saved for seed and the remainder is split equally between animal feed and industrial processing.

Oats are a temperate crop, more successful in wet climates than wheat or barley but less tolerant of cold. The majority of the oat crop is used in animal feeds. A variety of human food products are made from oats but these use only 5% of the world crop.

Rye is more resistant to pests, diseases, and cold than wheat and performs better on poor soils. Therefore, it tends to be grown on land just outside the belt giving the best wheat crops. Rye is the main bread grain of Scandinavian and eastern European countries, but worldwide only a third of the rye crop is used for human consumption.

28.2 MICROFLORA ON CEREALS IN THE FIELD

Cereals in the field are exposed to microorganisms from the soil, birds, animals, insects, manures, and from other plants in the field, particularly if diseased. A number of important fungal diseases of cereals may also be carried on the seeds themselves.[26] Thus, there is ample opportunity for microbes to contaminate the developing grains.

The most important factor governing the subsequent colonization of the grains, whether by saprophytes or pathogens, is the availability of water.[88] Hence, weather is important in determining the rate of microbial colonization of grain. Seiler[164] showed this quite clearly for wheat grown in the United Kingdom (UK) in 1976, a hot dry summer, and in 1977, an unusually wet one (Figure 28-2).

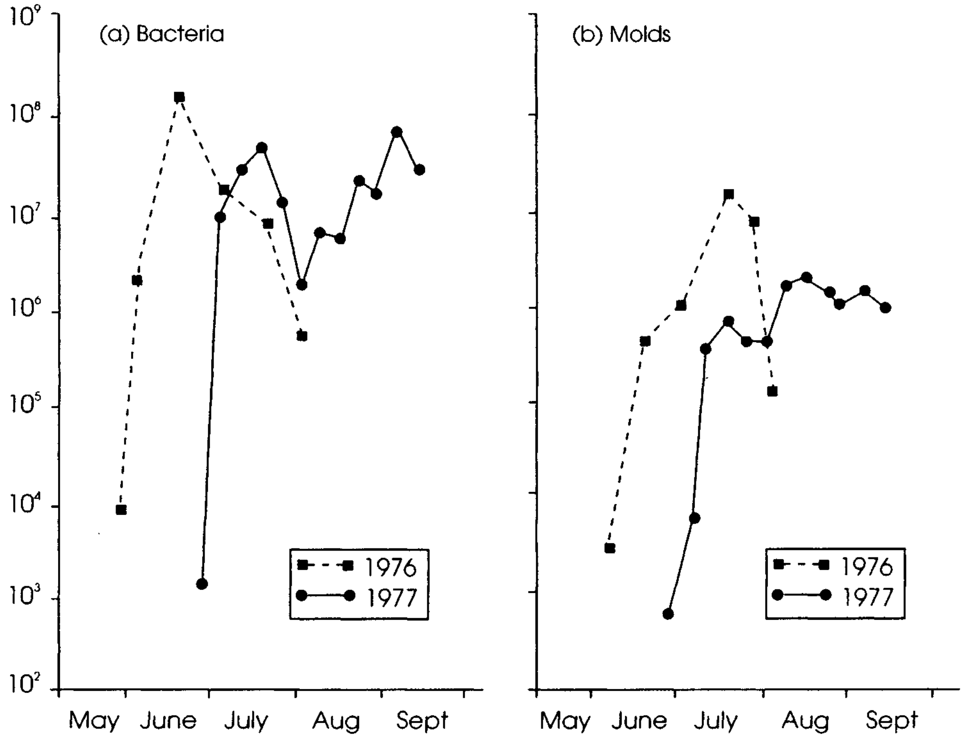

Figure 28-2 Numbers of Bacteria and Fungi on UK Wheat in the Field during Two Growing Years. The summer was long, hot, and dry in 1976, whereas the summer in 1977 was wet.

Peak numbers of both bacteria (*ca.* 10^8 g^{-1}) and molds and yeasts (10^6 to $10^7 g^{-1}$) were similar in both years. However, the populations peaked earlier in 1976 with a sharp decline over four dry weeks preceding the early harvest, whereas in 1977 the populations peaked shortly before the harvest.

Trojanowska collected cereal grains within a month of harvest in Poland for four years and the largest population of fungi occurred in 1980 when July and August were wet, with high average humidity and low sunshine.[189] The lowest population occurred in 1983 when average humidity was low and sunshine high, although there was some rain at the beginning of August. Populations on wheat, barley, and rye all followed similar patterns in relation to the weather. The relationship between weather and microbial population, however, is unlikely to be a simple one.

Microbes begin to colonize ripening grain soon after the ears emerge. Bacterial numbers rise first, closely followed by yeasts, yeast-like fungi, and filamentous fungi.[88] A bacterial population in excess of 10^8 g^{-1} has been observed on growing wheat.[164] Bacterial populations of 10^5 to 10^7 g^{-1} have been observed in wheat at harvest,[164] and on rice.

The yeasts most commonly isolated in temperate regions include *Cryptococcus laurentii*, *C. albidus*, *Rhodotorula (Torulopsis) ingeniosa*, *Sporobolomyces roseus*, and *Rhodotorula glutinis*.[88]

Many different filamentous fungi have been isolated from growing and newly harvested cereals in temperate regions including species of *Cladosporium*, *Alternaria*, *Verticillium*, *Eppicoccum*, *Fusarium* (including *F. culmorum*, *F. graminearum*, and *F. avenaceum*), *Botrytis*, *Dreschlera*, and *Monilia*.[88,189] Together these and other fungi that prosper in the high-moisture grain in the field are known as "field fungi." The colonization of cereals by individual fungi is highly variable (Table 28-1). *Alternaria alternata* has been detected in up to 100% of wheat kernels at the point of harvest.[28] *Cladosporium* spp. have been detected in over 90% of barley grains, even after a period of storage.[47] Colonization by *Fusarium* spp. is increased by rain and by "lodging," when the crop is knocked down by rain and wind and remains damp for a long period.

In warmer climates the predominant species are similar, but other fungi have also been found, including species of *Curvularia*, *Cochliobolus*, *Phoma*,

Table 28-1 Incidence of "Field Fungi" in Polish Grain in Harvest Years 1980–1982

	Percentage of Samples Contaminated											
	Wheat			*Barley*			*Rye*			*Oats*		
Genera of Fungi	*'80*	*'81*	*'82*	*'80*	*'81*	*'82*	*'80*	*'81*	*'82*	*'80*	*'81*	*'82*
Alternaria	0	20	23	5	13	0	0	23	9	0	0	23
Blastomyces	–	–	–	3	0	0	–	–	–	–	–	–
Botrytis	20	0	0	3	41	0	4	6	4	0	20	0
Cephalosporium	–	–	–	0	40	0	–	–	–	–	–	–
Cladosporium	0	30	47	13	26	54	8	4	19	14	6	54
Chalaropsis	–	–	–	–	–	–	0	9	0	–	–	–
Dreschlera	22	0	23	8	22	9	12	17	9	14	20	0
Fusarium	52	90	72	37	60	82	24	91	81	14	20	77
Monilia	–	–	–	–	–	–	0	3	0	0	26	8
Monosporium	–	–	–	0	6	0	–	–	–	–	–	–
Neurospora	–	–	–	0	11	0	–	–	–	–	–	–
Nigrospora	–	–	–	–	–	–	0	0	4	–	–	–
Pullularia	–	–	–	0	0	9	0	0	5	–	–	–
Scopulariopsis	–	–	–	0	13	0	0	3	0	–	–	–
Sporotrichum	0	0	6	–	–	–	–	–	–	0	0	46
Stemphylium	0	13	22	0	26	27	0	56	4	0	0	15
Trichoderma	–	–	–	–	–	–	0	17	0	–	–	–

Source: Data from K. Trojanowska, Evaluation of Cereal Grain Quality Using Mycological Methods, in *Cereal Grain Mycotoxins, Fungi and Quality in Drying and Storage,* J. Chelkowski, ed., pp. 185–215, © 1991, Elsevier Applied Science.

Penicillium, and *Aspergillus.* The last two genera would be considered primarily as storage fungi in temperate areas but appear able to colonize grains in the field when provided with higher temperatures and humidities. There may also be a change in the dominant species of each genus, eg, the dominant *Fusarium* species is more likely to be *F. semitectum* or *F. moniliforme,* particularly on maize.[88]

The presence of the majority of microorganisms appears to be of no consequence to the grain. A number of pathogenic fungi, however, can cause serious quality losses before harvest[12,26,59,76] or loss of crop yield and hence loss of value in trade.

More significantly, many species of fungi are known to be capable of producing mycotoxins on cereal grains in the field or in storage (see Chapter 53).[82,108] The first human disease to be associated with consumption of grains infected with a fungus was ergotism. Primarily associated with rye, the ergot fungus *Claviceps purpurea* can infect all the principal cultivated cereals to some degree.[100] From the middle ages to the late 19th century there were many epidemics of "St. Anthony's Fire" throughout Europe[18] particularly when a warm spring was followed by a wet summer. Improvements in farming and milling practices mean that ergotism is now considered a disease from the distant past. However, it is sobering to realize that a well-described epidemic occurred in the village of Pont-Saint-Esprit in southern France as recently as 1951[46] and outbreaks in developing countries were reported in the 1970s.[15]

In recent years attention has focused on other mycotoxins. In cereals in the field, aflatoxin produced by *Aspergillus flavus* or *A. parasiticus* has been found in maize[3,57] and sorghum[3] grown in tropical regions. Zearalenone produced by *Fusarium* species has been found in maize from the United States (US)[3] when conditions favored *Fusarium* ear rot and in wheat, barley, oats, and maize grown in New Zealand,[86] albeit at low levels. Fumonisins produced by *Fusarium* species have been found in maize in several European countries.[198] Deoxynivalenol (vomitoxin) has been identified in Canadian winter wheat[158,186] and in wheat from Kansas and Nebraska following harvest in years when the weather was warm and wet in May and June.[16,101] Hence, whenever conditions allow growing cereal crops to become infected with mycotoxigenic fungi, the risk of toxins being formed is present.

In practice, the presence of mycotoxins in cereals in trade is highly variable. In UK surveys during 1980–1982, 81% of samples of UK grown cereals (barley, wheat, and oats, total 345 samples) did not contain detectable levels of deoxynivalenol and only 4% contained more than 100 μg kg^{-1}. Over the same period, deoxynivalenol was not detected in only 16% of samples of cereals imported into the UK (maize and wheat) and 46% of samples contained over 100 μg kg^{-1}.[4] In surveys of the wheat harvested in the former USSR in 1986–1988, over 80% of samples examined contained detectable deoxynivalenol.[193] In a survey of the American wheat harvest of 1993, about 40% of samples contained deoxynivalenol at levels above the US Food and Drug Administration advisory levels for milling wheat.[191] Similar variability is found in relation to other mycotoxins including ochratoxin[110,195] and aflatoxin[195] with other cereals. Methods for detecting mycotoxins are now extremely sensitive and regulatory authorities have tended to be cautious, recommending limits for mycotoxins in foodstuffs, which creep down in line with analytical advances. It is difficult, therefore, to assess the level of concern that should be given to these results.

The association between weather and microbial activity in cereals, while undoubtedly complex, has led to attempts to identify factors that could be used to predict crop quality and/or disease outbreaks. Weather is known to affect grain quality and rain-damaged grain can have from 4 to 17 times as much α-amylase as undamaged grain.[39] A strong correlation has been shown between α-amylase content of wheat and counts of both bacteria and molds,[95] supporting the belief that wet weather contributes to poor microbiological condition of grain.

Legan and Curtis[95] and Neskorozhenyi and Zelenskaya[118] suggested, independently, that there is a statistical correlation between patterns of solar activity marked by the sunspot cycle and microbiological condition of cereals. Changes in solar activity are known to affect global climate; for example, sea surface temperatures vary with sunspot numbers over a double sunspot cycle.[119] It seems reasonable to assume that local weather conditions may, in turn, be affected, leading to variations in grain quality and microbiological condition. The solar cycle has a duration of approximately 11 years, so to confirm or refute these hypotheses over a number of cycles would involve work on a very long timescale.

28.3 HARVEST, DRYING, TRANSPORT, AND STORAGE

Regardless of the original source of the grain, the pattern of handling has certain similarities. The crop is cut, either mechanically or by hand. The harvest is threshed and winnowed to separate the grains from the chaff either within the mechanical harvester or using labor and the wind. The grains are dried in mechanical dryers using a forced flow of heated air, or using the sun and wind, unless the grain moisture content is low enough to ensure stability in storage. Finally, the grains are stored until sold or consumed; the storage facilities may be to a very high standard of hygienic design and insect proofing, or be no more sophisticated than storage on a barn floor. The storage period may bridge the seasons to the next harvest or be for up to several years in strategic reserves or European intervention "stockpiles." Transport is involved at several stages and may be local, from the field to the farm, or international, even intercontinental.

The fungal flora of the grains changes during postharvest drying and storage. The field fungi are adapted to growth at relatively high water activity (a_w), hence, high moisture content, and they die during drying and storage, to be replaced by a range of storage fungi that are capable of growth at lower a_w.

In 1,299 stored US export maize samples and 1,362 export wheat samples obtained in 1977 and 1978, the most frequently observed storage fungi were from the *Aspergillus glaucus* group. Essentially all maize samples yielded *A. glaucus*, typically in 15% to 30% of kernels, and 80% of wheat samples had detectable *A. glaucus*, typically in 1.5% to 4.0% of kernels.[155] *Penicillium* spp. were found in 7% of maize kernels and 0.1% of wheat kernels. *A. flavus* was frequently found in maize samples but typically only in a small percentage of kernels; whether this was due to preharvest or postharvest invasion was not clear. Other species of *Aspergillus* such as *A. candidus*, *A. restrictus*, *A. versicolor*, *A. ochraceus*, and *A. niger* were found in slightly less than 1% of maize kernels and about 0.1% of wheat kernels. *Cryptococcus albidus* was the yeast found most commonly in wheat samples from six regions of the US. Other yeast species found in wheat were *Cryptococcus laurentii*, *Rhodotorula glutinis*, *Pichia fermentans*, and *Pichia burtonii*.[83] However, only 26 of the 55 wheat samples examined yielded any yeasts.

Storage fungi of rice are primarily *Aspergillus* and *Penicillium* species but *Mucor*, *Fusarium*, *Cladosporium*, *Alternaria*, *Oidium*, and a *Sporendenema* species have also been detected.[31] *Penicillium* species were less prevalent on US rice than on Japanese rice, although no reason for this was apparent. Storage fungi on barley include *Absidia* spp., *Aspergillus glaucus* (group), and *Penicillium* spp.[47]

Fungi isolated in individual studies vary, particularly in the proportions of different fungal species in the population. However, the overall picture is of a storage mycoflora that is broadly similar at a genus level in different cereals from widely separated growing areas.[87,90,179]

Stored cereal grains also carry a bacterial population. The bacteria would be unable to grow in correctly stored grain because of the low moisture content (*ca.* 12% to 15%) giving a water activity of 0.7 or below, so it must be presumed that they represent the remnants of the population growing on the grains in the field plus any contaminants from soil and other causes encountered during growth, harvest, and storage of the grains. Counts of mesophilic aerobic bacteria in wheat range from *ca.* 10^4 to *ca.* 10^6 g^{-1}.[41,60,169] *Flavobacterium* spp. and *Pseudomonas* spp. were isolated from 91% and 82%, respectively, of US wheat samples tested.[60] *Brevibacterium*, *Bacillus cereus*, *Enterobacter cloacae*, and *Bacillus licheniformis* were isolated from less than 30% of samples tested. The numbers of each species present were not determined but the mesophilic aerobic bacterial counts were in the range 3.6×10^4 to -6.6×10^5.

28.3.1 Storage Conditions

Grain in storage must be protected from insects, which are responsible for a high proportion of grain damage,[143] and from fungal spoilage. Damaged grains are more readily colonized by storage fungi.[155] Once colonization has occurred, fungal growth is promoted by high water activity, and hence varies with grain moisture content. It has long been known that grain must be stored at low moisture content if it is to be stable for any length of time. For most grains, moisture contents in the range 10% to 14% are recommended,[40] the exact value depending on the grain type and desired storage life. In practice, the "safe" moisture content of the grain corresponds to that giving an equilibrium relative humidity (ERH) around the grain of 70% (or lower), equivalent to an a_w of 0.70.[19] For a fixed grain moisture content, the ERH rises by about 3% when the temperature increases by 10°C. This has serious implications for grains transported from temperate to tropical climates, which can experience a 20°C or more increase in temperature leading to an increase of 6% ERH (0.06 a_w), significantly increasing susceptibility to mold. Hence, for grain in international transport the loading temperature and likely temperature changes during transit must be considered as well as the moisture content.[109] The relationship between temperature, moisture content, and spoilage of maize (0.5% dry matter loss) is shown in Figure 28-3.

Initial moisture content of grain is not the only factor controlling stability, for water may migrate through the grain under the influence of temperature gradients. Water evaporates from grain in warm areas of the store and condenses in cold sections. Eventually, the local moisture content in the cool section may increase to a value permitting fungal growth. This may be a particular problem when the outside temperature is low relative to the grain temperature, because the natural insulating properties of the grain will retain heat within the center of a bulk store for extended periods. Cold air generated by con-

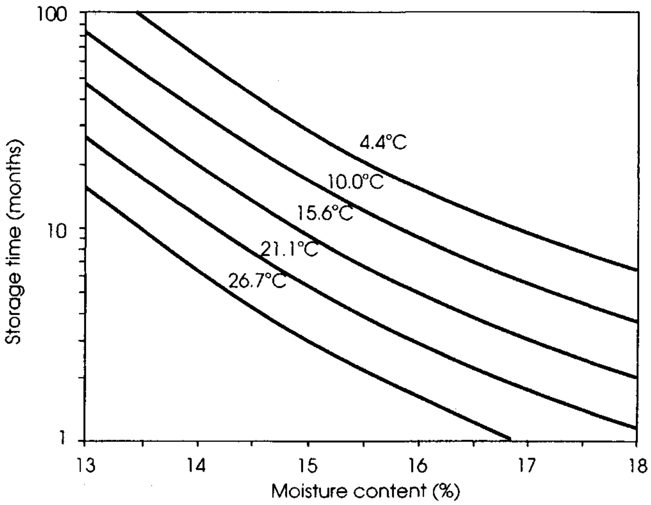

Figure 28-3 Allowable Storage Time for Maize before Spoilage (0.5% Dry Matter Loss) and Probable Loss of Grade Occurs. *Source:* Data from D.B. Sauer, Mold Invasion in Relation to Grain Damage, *Cereal Foods World*, Vol. 33, pp. 489–490, © 1988.

tact with cold bin walls passes down near the walls, into the center of the grain near the bottom, then rises, carrying moisture toward the top. The moisture is then deposited in the cooler surface layers of grain. Hence, ventilation of bulk stored grain is important. Elliot[40] describes the cycle of fungal growth, producing moisture and heat, prompting more fungal growth which can, in extreme cases, lead to spontaneous combustion of badly stored grain.

Molds growing on stored cereals produce a range of volatile odor compounds. Those identified that form a significant proportion of total odor volatiles include 3-octanone, 1-octen-3-ol,[192,206] 2-methylfuran, 2-methyl-1-propanol,[17] styrene,[206] and 3-methyl-1-butanol.[17,192,206] Sampling from the grain store ventilation system for fungal odor volatiles will provide a useful early warning of spoilage problems once the technology is robust enough to be used in such environments.

Storage of Wet Grains

Drying is expensive and can cause quality loss in the grains if not well controlled. Hence, it is no surprise that considerable effort has been invested into techniques for storing wet grain. These techniques fall into three main groups: temperature control, atmosphere control, and addition of chemical mold inhibitors.

Temperature Control. Cooling of wet cereals using refrigerated air has been used to prevent or delay fungal growth but even at temperatures close to 0°C, *Penicillium* spp. can cause spoilage of the grains if their moisture content is high enough. *Penicillium viridicatum* inoculated onto wheat was able to grow during storage at 18% to 20% moisture content and 6° to 12°C, even though other fungal species remained constant or declined in numbers.[115]

The total microbial load in wheat[172] and flour[199,200] can be reduced by storage at elevated temperatures provided that both temperature and holding time are carefully controlled to minimize damage to the wheat proteins. A 90% reduction in bacterial numbers in flour was achieved in 12 weeks at 28°C or 3 weeks at 37°C and a 99.9% reduction in less than 1 week at 48°C.[199,200] In wheat, bacterial numbers were reduced by 90% in 1 to 2 weeks at 44°C, 1 day at 50°C, and about 4 hours at 60°C.[172] It seems that the increased rate of reduction in bacterial numbers results directly from heat damage to the bacterial cells. This process has never found favor in commercial practice, being considered too expensive.

Atmosphere Control. Cereal grains may be stored in modified or controlled atmospheres to control stored-product insects. Typical atmospheres are 1% O_2 in nitrogen and 60% CO_2 in air. Both may be equally effective at controlling insects, but the high CO_2 atmosphere could be more effective at controlling fungal growth. A CO_2 concentration in excess of 15% is required to halve the linear growth rate of a number of field and storage fungi at 0.98 to 0.90 a_w and 23°C, with *Penicillium brevicompactum, Aspergillus fumigatus, A. nidulans,* and *A. versicolor* most sensitive to raised CO_2 of those fungi tested.[66] Mycotoxin production is more sensitive than fungal growth to raised CO_2 and to reduced O_2. However, fungal deterioration cannot be prevented completely in high-moisture cereals (a_w 0.80–0.90) as some fungi, particularly some *Fusarium, Mucor,* and *Aspergillus* species can grow in CO_2 concentrations of 60% to 80%. Yeasts and yeast-like fungi can also develop in high-moisture cereals in controlled atmospheres, causing rancidity and off-odors.[66]

Chemical Mold Inhibitors. Propionic acid is an effective agent for controlling growth of fungi in high-moisture grain and is recorded as preventing mycotoxin production by molds growing on high-moisture maize. Grains treated with propionic acid have the same, or slightly higher, animal feeding value than dried grains.[140] Propionic acid treatment has been used for many years at doses between 0.5% w/w in wheat at 15% moisture and 1.25% w/w in wheat at 30% moisture. There is some evidence that underdosing, whether accidental or deliberate, can result in the growth of fungal strains resistant to propionic acid, which then contribute to the fungal spoilage flora.[81] There is also some evidence that propionic acid and sorbic acids may stimulate aflatoxin production if present at subinhibitory concentrations.[128] More aflatoxin stimulation occurred in laboratory media when dilute (0.05% and 0.1% w/v) propionic or sorbic acid solutions were added to established cultures than when they were added with the fungal inoculum. The concentration of both preservatives in the cultures declined on incubation, indicating that they were metabolized by the *Aspergillus parasiticus* strain studied.

Other chemical treatments to inhibit mold growth or mycotoxin production have been investigated, particularly on high-moisture maize. These include various food grade phosphates,[89] sulfur dioxide with or without added ammonia,[38] ammonium and sodium bicarbonate,[114] sorbic acid,[128] and potassium sorbate with or without propylene glycol.[208] Many different treatments have the potential to inhibit mycotoxin formation, if not mold growth itself. Their disadvantages range from the cost of the treatment, which can be offset against the energy saving from reduced drying, to the corrosive effect of some treatments on the storage silos,[38] which is much more serious.

28.4 PRIMARY PROCESSING AT THE MILL

28.4.1 Cleaning

The mill is the point where cereals are transformed from agricultural commodities to foods or food ingredients. A great deal of physical cleaning takes place in the mill to remove straw and chaff, stones, soil, and metal fragments, which could damage the mill, damaged or ergot-infected grains, seeds other than the cereal grain required, insects, and other foreign matter. The various dry cleaning processes used in modern flour mills have little effect on the microbiological condition of the grain.[41,164,169] They are a considerable advance over the older washing processes, which had the potential to increase the microbiological contamination on the grain.[139] Cleaning processes are described in some detail by Kent and Evers.[73]

28.4.2 Conditioning

In dry milling of wheat, barley, rye, oats, and maize, it is necessary to raise the grain moisture content from that appropriate for safe storage to a higher value that improves the milling performance of the grain. This is achieved by a process called conditioning, or tempering, in which water is added to the grain, which is then held in a conditioning bin for the water to be absorbed. The rate of water addition and the holding time depend on the type of cereal. Holding times for most of the grain for milling would typically be only a few hours, so there is little opportunity for microbial growth to occur. However, conditioning equipment, like the rest of the mill, runs continuously and may only be cleaned weekly or even less frequently. Here, grain lodged in dead spaces may remain at high-moisture content for a prolonged period, promoting microbial growth and even grain sprouting. This grain can contribute to increased counts of molds, yeasts, bacteria, and, in particular, coliform bacteria in the conditioned wheat.[41] Some wheat millers add chlorine to the conditioning water in an attempt to reduce the amount of microbial growth that occurs at this point but little or no reduction of the bacterial count in the conditioned grain is achieved.[169]

28.4.3 Dry Milling

Characteristic features of milling processes are separation of the botanical tissues of the grain (eg, endosperm from pericarp, testa, and embryo) and reduction of the endosperm into flour or grits.[73] Milling may involve either or both operations: rice milling involves only separation, milling of white wheat flour involves separation and size reduction, and milling of wholemeal wheat or rye flours can be merely a size-reduction process.

In dry milling where separation is a key process, as in the production of white wheat flour, the result is flours containing substantially fewer microorganisms than the wheat in the milling grist.[41,151,164,169] This is simply because the microbes are primarily situated on the outside of the grain and are removed with the bran layers.[41,156] The bran is therefore more highly contaminated than the flour,[164] and contains similar or greater numbers of microbes than the milling wheat.[105,164] The microbiological condition of the flour output of the mill, however, remains highly dependent on the condition of the wheat input. This was shown most clearly by Oulsnam in a study of three mills in the UK (Figure 28-4)[126] in which white flour consistently contained approximately 1% of the number of bacteria and 17% of the number of molds and yeasts of the wheat.

28.5 FURTHER PROCESSING

28.5.1 Cereal Meals and Flours

The output from the mill in the form of various meals and flours is mainly used as a raw material for processing into a wide range of cereal products. The total microbial load of cereal flours is highly variable, reflecting the wide range of growing, harvesting, and storage conditions. Standard plate counts between 100 and 10^6 g^{-1} are not unusual, though 10^3 to 10^5 g^{-1} is the core range. Mold counts are a factor of 10 or so lower (Table 28-2),[44,68,69,137] and can also be highly variable.[70] In one study,[111] maize meal contained substantially more molds than either wheat flour or brown rice but whether this is truly representative is not certain.

Occasionally, yeasts may provide the dominant fungal population.[70] Yeasts found in flours from six regions of the US included *Hansenula anomala*, *Pichia farinosa*, *P. burtonii*, *Saccharomyces microellipsoides*, and an unidentified *Candida* species. The yeast-like mold *Aureobasidium pullulans* was also found. *Cryptococcus albidus*, which was the yeast most commonly found in wheat, was not

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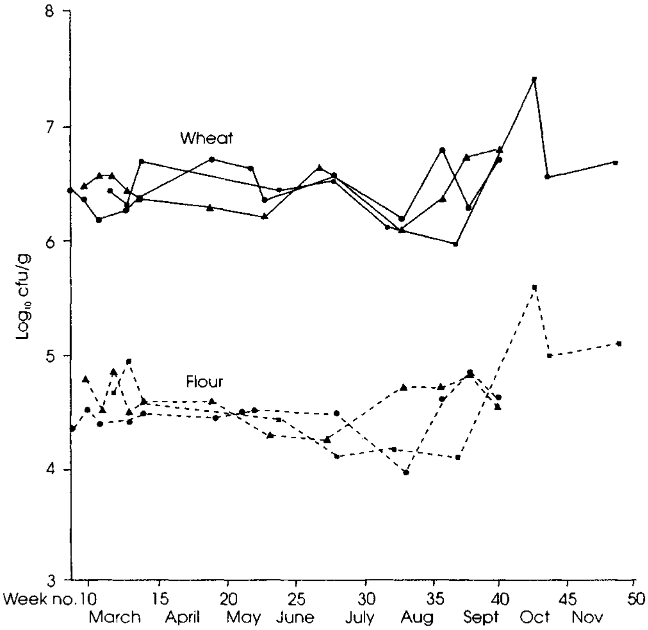


Figure 28-4 Numbers of Bacteria in Wheat Supplied to Three Mills and the White Flour Output from Them. The bacteria were enumerated using pour-plates of plate count agar incubated at 30°C for 3 days. The symbols (■, ●, ▲) each represent a different mill. Each result relates to a separate sampling occasion and hence to the condition of the material passing through the mills at that time. *Source:* Reprinted with permission from M. Oulsnam, Microbiological Condition of Breadmaking Wheat and Flour Taken from Three Mills during 1980, *FMBRA Bulletin,* pp. 179–185, August 1981, © 1981, Campden and Chorleywood Food Research Association.

found in any of the 60 flour samples tested. Overall, 22% of the flour samples yielded yeasts.[83]

Because no actively antimicrobial processes are used, it is not surprising that small numbers of coliforms[7,41,68,69,92] and *Escherichia coli*[68,69,92,149] are readily found in flours and brans,[72] or even that *Salmonella* can be found in a small proportion (0.3% to 2.3%) of flours.[149] This may be considered of little or no significance when the flour is to be used for baking or to make other cooked foods, but it should not be ignored as a potential source of cross-contamination.

In contrast, the presence in flours of spore-forming bacteria may be highly important. *Bacillus cereus* has been observed in a proportion (<10%) of both flours[41,68,69,92] and brans[72] at levels usually of only a few tens per gram. Other mesophilic aerobic spore-forming bacteria are common in flours at levels of up to a few tens per gram[68,69,92] and are a potential cause of spoilage of high a_w products, such as bread. Lactic acid bacteria have been found in over 90% of wheat flours[50,68,69,92] at levels up to 10^4 g^{-1}, though commonly <1,000 g^{-1}, and may contribute to the spoilage potential of uncooked cereal products such as chilled pastry.

Some flours for special purposes are made from grain that has been heat treated using steam to alter the functional properties of the flours. Not surprisingly, these flours contain fewer microorganisms than normal flours. However, in the event of a process breakdown leading to hot, moist grain being held for a prolonged period of time, the number of thermophilic spore-forming bacteria may increase dramatically.

28.5.2 Doughs and Batters

Doughs and batters are an intermediate stage in the manufacture of bread, cakes, pastries, and other cooked cereal products. Essentially, the ground cereal is mixed with water and other required ingredients. The difference between a dough and a batter is one of viscosity: a batter will flow and pour, albeit viscous batters may flow only slowly, but a dough is more resilient. Batters and doughs may be aerated by microbial fermentation, chemically, or not at all. Either may be enriched with sugars, fat, fruit, or other ingredients depending on the final product desired.

Rye and Mixed-Cereal Sourdoughs

The earliest fermented doughs were probably sourdoughs, which developed naturally through growth of lactic acid bacteria and yeasts from the flour. Such spontaneous sourdoughs have become fashionable for production of "organic" rye breads.[136] Sourdoughs retain a dominant position in the manufacture of rye breads because the water-soluble proteins of rye flour do not form gluten. Hence, dough structure relies on pentosans and "mucilages" whose contributions are enhanced in an acid environment; therefore, dough made from flour, comprising more than 20% rye flour, must be acidified to make acceptable bread.[150] The technology of sourdough bread production is described in detail by Spicher.[176] Typically a starter or seed sour is used consisting of a mixture of sourdough bacteria at levels between 2×10^7 and 9×10^{11} g^{-1} and yeasts at 1.7×10^5 to 8×10^6 g^{-1}.[176] The starter may be obtained from a commercial supplier or maintained in the bakery by feeding it with flour and water at intervals. Some bakery-maintained starters could be surprisingly old, possibly 100 years old or more in the case of some San Francisco sourdough starters.[78,182]

Table 28-2 Average Numbers of Some Microorganisms in White Flour Produced in the UK from New-Harvest Wheat (expressed per gram of flour)

Year of Harvest	*No. of Samples Tested*	*Bacteria*[1]	*Molds and Yeasts*[2]	*Spores of Mesophilic Aerobic Bacteria*[3]	*% Samples Containing*	
					Coliform[4] *Bacteria*	Escherichia coli[5]
1988	53	110,000	2,700	56	100	40
1989	43	42,000	1,500	20	72	28
1991	45	369,000	3,080	60	98	53
1992	41	102,000	3,200	125	95	56
1993	55	410,000	4,900	93	89	55
1994	66	97,000	1,350	71	92	44

[1] Enumerated by the spiral plate technique on Cysteine Lactose Electrolyte-Deficient (CLED) medium incubated at 30°C for 3 d. This medium was used to reduce colony spreading, hence making the plates easier to count.

[2] Enumerated on Oxytetracycline glucose yeast-extract medium incubated at 25°C for 5 d.

[3] Enumerated by dispersing 5 ml of a 10^{-1} suspension of flour in 100 ml of molten (45°C) CLED medium, heating in a waterbath at 80°C for 34 min, cooling rapidly to 45°C and pouring 5 plates. Once set, the plates were given a thin overlay of CLED medium and incubated at 30°C for 3 d (counted after 2 and 3 d).

[4] Enumerated by a most probable number (MPN) method using Lauryl Tryptose Broth (LTB) at 37°C for 24 h with positive tubes subcultured to Brilliant Green Bile Broth (BGBB) at 37°C for 24 h. The MPN was derived from the BGBB tubes showing growth and gas production.

[5] Positive BGBB tubes form the coliform test[4] were subcultured to BGBB and Tryptone water tubes incubated at 44°C for 24 h. The MPN was derived from the subcultures showing growth and gas production in BGBB and indole in tryptone water.

Source: Reprinted with permission from J.D. Legan, The Microbiological Condition of 1994 Harvest Flours, *Chorleywood Digest*, pp. 112–113, December 1994, © 1994, Campden and Chorleywood Food Research Association.

Fourteen different identified species of *Lactobacillus*[99,122,150,176] (Table 28-3) and a range of unidentified *Lactobacillus* spp.[98] have been isolated from starters and sourdoughs as have *Leuconostoc dextranicum*, *Pediococcus* spp., and members of the Enterobacteriaceae.[99]

Eight different identified yeast species have also been isolated from sourdoughs or starters (Table 28-3) including species of *Saccharomyces*,[55,176,182] *Hansenula*,[55] *Pichia*,[176] *Candida*,[150,176] and *Torulopsis*.[176] Although it appears that more bacterial species than yeast species are involved in sourdough fermentations, one should not forget that more attention has historically been paid to the bacteria present in sourdoughs than to their yeasts. There are probably more sourdough yeasts still to be identified.

Yeasted Wheat Doughs

The majority of the world's leavened wheat breads are produced from yeasted doughs containing *Saccharomyces cerevisiae*, added by the baker when mixing the dough. Bakers originally obtained the surplus yeast from their local brewery. However, modern bakers' yeast strains have been developed for desirable characteristics such as rapid gas production and are produced commercially in dedicated fermentation plants. Even so they typically contain some lactic acid bacteria that may be important in flavor development.[181] The technology of bakers' yeast production has been reviewed by several authors.[24,187,188]

Traditional yeasted-dough processes involve a bulk fermentation stage of about 3 hours, although in many small bakeries a 16-hour (overnight) fermentation, known as a sponge, is used as a convenient way to shorten or eliminate the bulk fermenta-

Table 28-3 Microorganisms That Have Been Isolated from Sourdoughs and Sourdough Starters

Species	*Reference*	*Species*	*Reference*
Bacteria			
Lactobacillus acidophilus	176	*L. sanfrancisco*	150
L. casei	176	*L. reuteri*	122
L. plantarum	176	*L. brevis*	122
L. farciminis	176	*L. hilgardii*	122
L. brevis	176	*L. curvatus*	122
L. buchneri	176	*Pediococcus* spp.	99
L. fermentum	176	*Leuconostoc dextranicum*	99
L. fructivorans	176		
L. alimentarius	99	Enterobacteriaceae	99
Yeasts			
Saccharomyces cerevisiae	182	*Pichia saitoi*	176
S. exiguus	55	*Candida crusei*	176
S. inusitatus	182	*C. milleri*	150
Hansenula anomala	55	*Torulopsis holmii*	176

tion. The sponge can be linked to short-time dough processes such as the American sponge and dough method or the Rabinovitch continuous bulk fermentation used in the former USSR.[29] Alternatively, no-time mechanically developed dough processes, such as the Chorleywood Bread Process, may be employed. During the bulk fermentation and sponge and dough processes, flavor compounds, including organic acids and diacetyl, are produced.[181] In mechanically developed doughs, the only fermentation occurs during the short proof time, which may be less than an hour long, when the dough is held warm in a humid atmosphere. During this process step, the yeast produces gas and causes the dough to rise, increasing in volume to give the finished loaf an open crumb structure and light texture.

Yeasted doughs are only slightly acidic, mainly as a result of dissolved carbon dioxide, which is driven off during baking, so they do not provide as much resistance to the growth of undesirable bacteria as sourdoughs. Observations within bread plants, when processes have gone out of control leading to spoilage problems, have shown that *Bacillus* spp. can reach high populations in scrap dough. Unfortunately, the age of the scrap is usually not known, making a judgment on the rate of *Bacillus* growth in the dough impossible. There appear to be no published observations of the growth of pathogenic bacteria in yeasted doughs.

Batters

Simple batters, such as those used for coating foods, consist of little more than cereal flour and water. They have a high a_w and their microflora initially reflects that of the components of the batter. These batters may be held for extended periods, being pumped over the food to be coated with the excess batter recycled. This puts a great deal of emphasis on the need for effective control of batter temperature and maximum holding time. Even in plants operating within the US Food and Drug Administration Good Manufacturing Practice Regulation, batters have been found to contain large numbers of pathogens and other bacteria (up to 5,000 *Staphylococcus aureus* and/or 93,000 *Escherichia coli* per gram).[36] That this does not simply represent contamination of the batter by the food being coated is illustrated by the observation that the bacteriological condition of shrimp in 30 US processing plants improved during processing, but the geometric mean coliform load roughly doubled on battering.[36]

Batters for production of crumpets and certain other hotplate goods contain bakers' yeast, added for flavor and gas production. The potential for *S. aureus* to grow in these is lower than that in simple batters, presumably as a result of competition from the yeast.[127]

Cake batters may contain a wide range of ingredients in addition to cereal flour, for example, fats, egg, sugar, fruit, and others. Hence, the potential range of microbes present, including pathogens, is increased. However, the reduced water activity and relatively short holding time before baking restrict the opportunities for microbial growth.

Other Doughs and Batters

A range of traditional fermentations are used to make products including puto, jeungpyon, and tape (rice cakes from the Philippines,[116] Korea,[71] and Indonesia,[30] respectively); kisra, a Sudanese sorghum flat bread[113]; kenkey, a fermented maize dumpling from Ghana[120]; and ogi, a West African infant food made from maize and sorghum.[153] These products have been developed empirically over generations to suit local conditions and the majority involve primarily a lactic acid fermentation but a detailed understanding of their microbiology is still in its early stages.

28.5.3 Baked Products

Bread

Bread is one of the most important dietary staples in the world and there are a great many national and regional variations on basic types, as well as special breads such as malt loaves, soda breads, milk breads, and fruited breads. In 1991, the value of the Western European bread market was estimated at 230,000 million French francs.[13] Bread is prepared from a dough of cereal flour, which may be unleavened or fermented using yeasts or using a mixture of lactic acid bacteria in a sourdough, and baked. Typical formulations for wheaten bread are shown in Table 28-4.

Microbiologically speaking, the most significant property of bread is its high moisture content (*ca.* 40%) and a_w (*ca.* 0.94–0.97). Bread is baked at oven temperatures between 190° and 260°C (typically about 220°C) but the center crumb temperature does not exceed about 97°C and then only for a few minutes at the end of baking. This makes bread susceptible to growth of molds, which recontaminate the loaf on cooling, and certain bacteria, particularly spore-forming *Bacillus* species that survive the baking process. It has only a short shelf life of 3 to 7 days unless special steps are taken. Few estimates of the losses of bread attributable to mold spoilage in developed countries are available but figures in the region of 1% to 5% have been suggested.[67,75] Mold spoilage

Table 28-4 Formulations for Some Typical Wheaten Breads in the UK,[20] Relative Quantities of Different Ingredients

	Chorleywood Bread Process (CBP)		*Bulk Fermentation*	
Ingredient	*White*	*Wholemeal*	*White*	*Wholemeal*
White flour	100	–	100	–
Wholemeal flour	–	100	–	100
Water	60	62.5	58	60
Yeast	2.1	2.5	1.6	2.1
Salt	2.1	2.1	2.0	2.1
"Compound improver"[a]	1.0	1.0	–	–
Fat	–	1.8	1.1	1.8
Soya flour	–	–	0.7	–
Malt flour	–	–	0.2	–

[a] One of a number of proprietary products to help dough development in the CBP process.

Source: Reprinted with permission from J. Brown, Recipes and Processing Methods, in *The Master Baker's Book of Breadmaking*, pp. 182–299, © 1982, The National Association of Master Bakers, Confectioners and Caterers.

of bread and methods for its control have been reviewed by Legan.[91] Wheat breads, such as those consumed in Western Europe, North America, and elsewhere are usually yeast raised and typically have a pH of about 5.4 to 6.0. These breads are susceptible to bacteriological spoilage caused by *Bacillus* species, known as "rope," as well as to mold growth. In Germany, Scandinavia, and many eastern European countries, rye breads made by a sourdough fermentation process are popular. These breads are much more acid than wheat breads, having pH in the range of 3.5 to 4.8 with a total acidity (sauergrad) in the range of 20 to 8.0 ml.[176] The total acidity is the number of milliliters of 0.1 M NaOH needed to titrate the acid in 10 g of bread to neutrality against a phenolphthalein indicator. If the sourdough fermentation produced only lactic acid this would be equivalent to 2.2% to 0.9% lactic acid.

Despite the fact that bread is distributed at ambient temperature and known to be susceptible to microbial growth, it has a very good public health record. However, bread has been associated with outbreaks of foodborne disease, even in recent years. In 1989, there was an outbreak of hepatitis A in Cambridgeshire, UK, in which the vehicle of transmission for the virus appeared to be unwrapped bread sold from a small shop staffed by an infected food handler.[203] In this case the food vehicle could just as easily have been any other unwrapped food but the case serves as a useful reminder of the need for basic food hygiene, even when handling products not normally considered particularly high risk. There have also been a small number of cases of food poisoning, caused by *Bacillus* species other than *B. cereus*, associated with the consumption of bread spoiled by rope.[25]

Mold Spoilage. Mold spores are killed by the baking process,[79] hence molds spoiling bread gain access during the cooling, slicing, or wrapping operations. The spores are generally considered to spread through the air, with airborne mold spore counts between 50 and 5,000 m^{-3} having been recorded in bakeries.[123,162,175] It is likely that airborne molds are associated, at least in part, with the spread of flour throughout the bakery.[52] Airborne mold spore counts within the bakery are higher when activities such as mixing or cleaning are in progress and when there are more molds in the outside air. High counts of airborne molds are associated with the formation of relatively high numbers of mold colonies on each loaf of bread stored for long enough for spoilage molds to become visible. This relationship breaks down with low airborne counts, suggesting a residual level of contamination from surfaces, equipment, and handling (Figure 28-5).[162]

New strains of molds are continually introduced into bakeries from raw materials and the environment.[207] Nevertheless, conditions within a particular bakery are reflected in the incidence of molds on its products.[121]

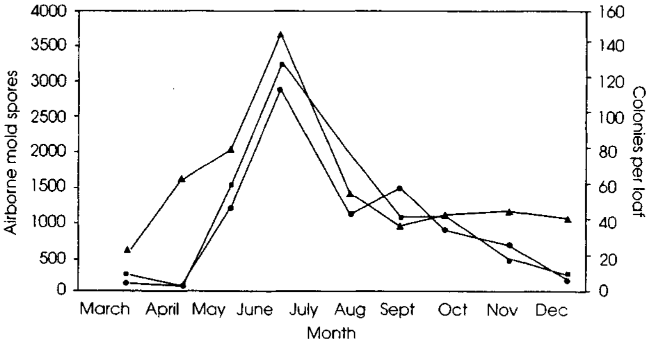


Figure 28-5 Relationship between Airborne Mold Spore Counts and Mold Colony Counts on Bread. ■ Mold spores in air outside the bakery, ● mold spores in air in the bread cooling area, ▲ colonies forming on bread. The bread was stored at 27°C for 6 days before examination. *Source:* Reprinted with permission from D.A.L. Seiler, Counting Moulds in the Bakery Atmosphere, 1. Seasonal Fluctuations, *FMBRA Bulletin*, pp. 54–58, April 1972, © 1972, Campden and Chorleywood Food Research Association.

The most common bread spoilage molds are *Penicillium* spp.,[2,63,121,131,176,177] although *Aspergillus* spp. may be more important in tropical countries.[129] On loaves of yeast-raised, wheat breads made and stored in the UK until visibly moldy, Odell[121] observed *Penicillium* spp. on 90% to 100% of loaves, *Aspergillus* and *Cladosporium* spp. on about half the loaves, and Mucorales on 20% of the loaves. The incidence of the different genera was constant throughout the year. The type of spoilage appearing first on sourdough rye breads in Germany was dominated by *Penicillium roquefortii* (91.4% of 437 loaves stored), other penicillia (3.4%), and *Aspergillus* or *Eurotium* spp. (3.4%).[177]

Mycotoxigenic species of *Aspergillus* and *Penicillium* have been isolated from bread.[23,185,197] Moreover, mycotoxins including citrinin,[144] aflatoxins B_1, B_2, G_1, G_2, and M_1,[145,146] ochratoxin A,[145] and a range of *Alternaria* toxins[147] have been detected in inoculated breads. However, even in inoculated breads not all mycotoxigenic molds are able to produce toxin.[144] Some mycotoxins, eg, patulin and penicillic acid, are inactivated in the presence of bread proteins,[124,144,148] and mycotoxins do not readily diffuse through bread in the absence of hyphal ramification.[145] Moreover, mycotoxins are only detected after bread shows visible growth of molds. These factors contribute to the low risk to consumers in developed countries from mycotoxins in bread. The picture is not so clear in developing countries. There is a statistical correlation between cirrhosis of the liver and consumption of moldy steamed bread in China[22] but other risk factors such as hepatitis and pipe smoking were also identified.

"Rope" and Other Bacterial Spoilage. The spoilage condition of bread known as "rope" is caused by the growth of *Bacillus* spp. The rope organism was first identified as *B. mesentericus* by Watkins in 1906[204] but later renamed as *B. subtilis*. Subsequently, other *Bacillus* spp. have been identified in ropy bread, especially *B. licheniformis* and occasionally *B. pumilis*, *B. megaterium*, and *B. cereus*.[77]

Bacillus spores are present in flour[11,64,68,69,74,92,97] and other ingredients[64] and may also be found on bakery equipment,[201] particularly when hygiene does not receive enough attention. They are resistant to the heat process given in baking and survive in the center of the loaf. Once the bread has cooled, the spores germinate and grow. Rope is noticed first by its odor, usually described as "fruity" or "sweet" like overripe melons or pineapple but the description is rather subjective. It has been described as "objectionable," resembling "a mixture of rotting fruit and bad cheese."[45] As the condition develops, the center of the loaf becomes sticky until fine threads can be drawn from it. The crumb subsequently darkens and, once the condition becomes extremely advanced, may collapse completely.

Rope is of limited economic significance in temperate countries, where growth of the bacilli is constrained by a combination of preservatives and moderate ambient temperature allowing molds to become visible before rope spoilage develops. Nevertheless, outbreaks causing losses to individual bakeries are experienced during most summers. However, in hot countries such as South Africa, rope is a significant cause of loss.[77] Moreover, it has been recognized in recent years that *B. subtilis* and *B. licheniformis* both have the potential to produce a toxin-mediated food poisoning.[196] Hence, even in temperate countries this adds emphasis to the need to prevent rope spoilage. Rope is not a problem in sourdough rye breads where it is prevented by the acidity of the bread.

"Bleeding bread" is a spoilage condition caused by growth of *Serratia marcescens* when ambient temperatures are high. This results in production of red or pink pigment such that heavily affected bread appears to bleed. Early examples were attributed to miracles.[183] Bleeding bread is rare nowadays, presumably as a result of improved bakery hygiene, combined with the use of preservatives.

Preservation. Bread preservation aims to delay the onset of both mold and rope spoilage, reducing losses and giving extra shelf life to the product. The principal bread preservatives in use worldwide are propionic, sorbic, and acetic acids as well as certain of their salts. Background information about these materials is given by Lueck[102] and Doores[35] and a detailed review of sorbates has been given by Sofos and Busta.[174] The use of preservatives is regulated by law in many countries and reference should always be made to the appropriate legislation when deciding on a preservative regime. In the UK, the preservative of choice for all bread for many years has been calcium propionate, but recent legislation has restricted its use to prepacked breads.[5]

Propionates are commonly used in bread as 0.1% propionic acid or 0.2% calcium propionate based on flour weight,[91] which gives about one extra day's life to yeasted wheat breads. These materials inhibit both molds and rope but also reduce the activity of the yeasts in the dough, necessitating an increased yeast level, longer holding (proof) time for the yeasts to produce gas, or use of an improver such as ascorbic acid to avoid loss of loaf volume.[91]

Sorbates have greater antimold activity on a weight-for-weight basis than propionates. Hence, they cause greater inhibition of the yeast. They can also make doughs very sticky and difficult to process.[91] Sorbates are widely used in German sourdough breads[177,178] at levels not exceeding 0.2% of final product weight. Presumably, the longer fermentation times and mixed ferment of yeast and lactic acid bacteria make the inhibition of yeast activity less noticeable in these products. In the US, breads have been successfully preserved against molds using a spray of sorbic acid[58] or potassium sorbate[75] applied after baking.

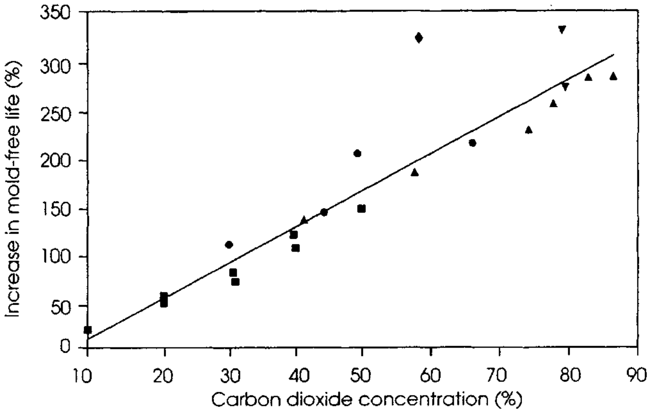
Acetic acid, in the form of vinegar, is used traditionally to prevent rope.[9] A concentration of 0.1% to 0.2% w/w of acetic acid in flour is effective against rope but has a limited effect on molds. At higher levels, the odor of vinegar becomes quite noticeable.[91] Sodium diacetate (sodium hydrogen acetate) at 0.3% w/w of flour will retard molds under most practical conditions.[53] Calcium acetate at 0.3% w/w of flour was more effective than 0.2% calcium propionate for inhibiting rope.[77]

Ethanol, where permitted, increases the mold-free life of bread when used at concentrations between 0.5% and 3.5% applied after baking.[51,136,167,171,202] The benefit is increased because it is one of the few materials that can also retard staling.

There has been considerable interest in ingredients with "natural" antimold properties. Dried, cultured wheat flour containing lactic and acetic acids has been used in the US.[117] Also in the US, raisins[37,85,209] and prune juice concentrates[152] have been shown to extend the mold-free life of bread. Hydroxymethyl furfurals formed from the fruit sugars during baking, together with naturally occurring tartaric and propionic acids, probably account for the antimold activity, though this has yet to be proven.

Modification of the atmosphere around the product using a high concentration of carbon dioxide gas retards the growth of molds on bread and other baked products (Figure 28-6).[21,34,80,94,103,167,168,173,202] Mold-free life can be increased by 100%, 200%, and 300% in atmospheres containing respectively 40%, 60%, and 90% CO_2 by volume. However, CO_2 at atmospheric pressure does not inhibit yeasts to any significant extent. Hence, bread packed in CO_2 atmospheres shows a higher incidence of the spoilage type known as "chalk mold," caused by yeasts that show mycelial or pseudomycelial growth, especially *Pichia burtonii* and *Saccharomycopsis fibuligera*. CO_2 does not prevent the growth of *Bacillus licheniformis*; therefore, in time, CO_2-packed breads may develop high populations of *B. licheniformis* or even display signs of rope spoilage, particularly in hot weather. Questions have also been raised about the potential for anaerobic spore-forming bacteria to grow in CO_2-packed breads. During the long history of these products in the marketplace, there have been no recorded problems of this type, but the factors preventing them are not clear.

Gas-packaging equipment is expensive. Hence, breads packed in CO_2 tend to be high-value niche products such as special dietary breads, ethnic breads, or long-life part-baked breads where reheating by the consumer is used to reverse the effects of staling. Another approach to atmosphere control, requiring less expensive equipment, is to use oxygen-absorbing materials such as "Ageless" (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan) or the ATCO oxygen absorber (ATCO S.A., Caen, France) to reduce the oxygen concentration in the pack.[84,138,173] Powers and Berkowitz[138] showed that this method can keep bread free of mold growth for over a year; however, they did not investigate the potential for growth of anaerobic spore-formers.



■ Bread packed in glass containers[168]; ● bread and ▲ crumpets in commercial packs[170]; ▼ bread[202]; and ◆ crusty bread rolls.[179]

Figure 28-6 The Extension in Mold-Free Shelf Life of Bread and Crumpets in Atmospheres Containing Different Concentrations of CO_2. Reprinted from International Biodeterioration and Biodegradation, Volume 32, J.D. Legan, Mould spoilage of bread: the problem and some solutions, Copyright 1993, pages 33–53, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington, OX5 1GB, UK.

A model for growth of *Clostridium botulinum* in cooked vacuum packed potatoes predicts that growth and toxin production can occur at 25°C, pH 5.50, and a_w 0.965,[33] conditions that could easily be achieved in bread products made from yeasted doughs. It is more difficult to compare the redox potential in cooked vacuum packed potatoes with that of breads in modified atmosphere packs because measuring the redox potential inside a food product is fraught with many difficulties. In an analysis of 2,000 outbreaks of botulism between 1951 and 1989 (most after 1980), no involvement of flour-based products was found.[65] Thus, there is no evidence for a real botulism hazard from bread products packed in modified atmospheres, perhaps because the packs have contained sufficient residual oxygen to inhibit growth and toxin formation. High-speed gas-flushing equipment typically achieves 90% atmosphere replacement when packaging bread and cakes, implying a residual oxygen concentration of about 2%, whereas residual oxygen concentrations well below 1% can be achieved in vacuum packs. Nevertheless, the factor(s) governing the safety of bread products packed in modified atmospheres are not currently well understood. Not enough is known about how interactions between residual oxygen concentration, pH, and water activity prevent the growth of *C. botulinum*.[205] It is not safe to assume that merely because some products have not caused botulism in the past they will not do so in future.[1]

As with other food products, the best way to ensure the safety of cereal products in modified atmosphere packaging is to adopt a hazard analysis critical control point (HACCP) approach.[94,112] *Clostridium botulinum* would not be the only concern of a HACCP system applied to bread products packed in modified atmospheres but it may easily be overlooked. The minimum a_w for growth of *C. botulinum* types A and B is 0.94.[49] It is difficult to consistently manufacture a palatable bread with an a_w lower than this, so a combination of pH, a_w, and preservatives is most likely to be used to prevent the growth of *C. botulinum* in these products.

Flour Confectionery

Flour confectionery (eg, cakes, pastries, puddings) includes an intriguingly wide range of microbiological problems. At one extreme, the products can be extremely perishable, usually as a result of the inclusion of a filling such as cream or custard with a high a_w. Such products can support bacterial growth and have been associated with outbreaks of food poisoning. At the other extreme the products may be stable for months or years as a result of intermediate or low a_w.

Association with Food Poisoning. Out of 2,226 outbreaks of food poisoning in the US between 1973 and 1987, 51 were attributed to bakery products. The split between different types of baked goods was not reported, nor was the contribution of any filling.[14] It is clear, however, that cakes and pastries with perishable fillings, such as cream or custard, have been the cause of food poisoning. These products are often produced in small craft or artisanal bakeries where a high proportion of hand operations is involved in filling and finishing the product. Therefore, it is not surprising that in surveys published since 1983, coliforms[107] and *E. coli*[61,133] have been observed in up to 30% of cakes and pastries, particularly those filled with cream. The incidence of *E. coli* was worse in dairy cream slices (23 out of 92 products) than in nondairy cream slices (12 out of 101 products).[61] *Staphylococcus aureus* and *B. cereus* have also been found in between 4.0% and 25% of cream-filled pastries.[61] Even in reasonably large plant operations operated to US good manufacturing practice guidelines, coliforms were detected in nearly 30% of cream pie fillings just prior to freezing.[157] However, the numbers present were low, with 97.5% of samples yielding <150 g^{-1} and the incidence of *E. coli* was close to zero.

An example of outbreaks associated with these products is an outbreak of food poisoning in 1983 linked to custard-filled vanilla slices; 36 people suffered diarrhea and vomiting in 15 minutes to 3 hours after consuming the product.[43] *S. aureus, B. cereus,* and *B. subtilis* were all isolated from vanilla slices taken from the same batch as those causing the outbreak. The primary cause of illness was attributed to *S. aureus* enterotoxin A, but the rapid onset of illness suggested that *B. subtilis* or *B. cereus* toxin(s) may also have been involved. In another outbreak in 1991, 17 people contracted *Salmonella enteritidis* phage-type 4 food poisoning from custard slices from a small bakery.[10] The custard had been made with fresh shell eggs and had not been properly cooked.

The potential for pathogens to grow in cakes and pastries, particularly those with cream or custard fillings and/or high water activity, and cause illness in consumers emphasizes the need to operate strict hygienic controls during manufacture of these products. Moreover, it demonstrates the need to take steps to control growth of organisms between manufacture and consumption of the products. This is most easily done by chilled or frozen storage and display. Unfortunately, chill temperatures may accelerate the rate at which the cake or pastry stales. In UK law, perishable foods at or in food premises are now required to be kept below 8°C, although there is a derogation to allow the sale of such foods at room temperature for a maximum of 4 hours after production, after which they must be destroyed.[6] As a result it is only in small craft bakeries that it is still possible to buy custards, sandwiches, and similar products at ambient temperatures.

Spoilage. Flour confectionery products with high a_w may be susceptible to bacterial spoilage, the most familiar manifestation being the souring of dairy cream fillings by lactic acid bacteria. Similar spoilage may occur in raw pastry during chilled storage, particularly if the numbers of lactic acid bacteria are allowed to increase during manufacture, for example, by poor control of recycled trimmings or poor temperature control.

More commonly one thinks of "ambient-stable" flour confectionery products. These are expected to have shelf lives of anything from a few days to several months and are generally very safe because their a_w is too low to support bacterial growth. In most flour confectionery products the primary factor limiting shelf life is mold or yeast growth. However, rancidity, staling, drying out, or softening due to moisture gain are all factors capable of limiting the life of these products.

The rate at which molds or yeasts spoil flour confectionery is defined in the first instance by the a_w of the product.[160,163,165] Typical a_w and spoilage for a range of products is given in Table 28-5. Other important product characteristics are pH and type and concentration of preservatives. Environmental conditions including temperature and atmosphere around the product will also influence the rate of spoilage.

Mold or yeast spoilage of flour confectionery could manifest itself in several ways[93]:

1. As typical mold colonies, especially *Penicillium* or *Aspergillus* spp., but in products with lower a_w, xerotolerant molds, including *Eurotium* spp. and *Wallemia sebi*, become more common.
2. As bubbling in jams, fondants, or fruit fillings or as pitting or cracking of icings as a result of the pressure of carbon dioxide gas formed by yeast fermentation. Fermentation also produces alcohol and other compounds with strong odors. For example, *Pichia anomala* can produce ethyl acetate, which may give the impression of a product suffering from a chemical adulteration. Another yeast, *Pichia burtonii*, has been known to produce styrene when fermenting spiced syrup containing cinnamon[27,96,132] as used for glazing hot-cross buns.
3. As low white or off-white "dusty" growth of one of a number of "pseudomycelial" yeasts such as *Candida guilliermondii, C. parapsilosis, Debaromyces hansenii, Pichia anomala, P. burtonii,* or *Saccharomycopsis fibuligera* on the product surface. This is particularly visible on the surface of dark products and is known as "chalk-mold" because of its resemblance to a sprinkling of chalk dust.[96] It is more frequently seen on breads than on flour confectionery.

In products with a_w below 0.8 the spoilage mycoflora becomes restricted to the more xerophilic molds and osmophilic yeasts. Below a_w 0.7, flour confectionery can, for most practical purposes, be considered safe from spoilage, provided that condensation is avoided as it can lead to localized regions of higher a_w. However, there are a few species able to grow at a lower a_w, notably the yeast *Zygosaccharomyces rouxii*, minimum a_w 0.65,[184] and the mold *Xeromyces bisporus*, minimum a_w 0.61.[134] Hence, on rare occasions, molds or yeast may spoil flour confectionery with a_w lower than 0.7. Products with a_w below 0.6, such as biscuits and crackers, are not at risk from microbiological spoilage provided that they are packaged and stored to prevent moisture migration from the environment or within the product leading to a general or localized increase in a_w.

Table 28-5 Water Activity of a Range of "Cereal" Products and the Principal Type(s) of Spoilage Associated with Them

a_w	*Products*	*Spoilage Types*
0.99	Creams, custards	Bacterial souring
0.95–0.98	Breads, hotplate goods	Bacterial spoilage, eg, "rope"; mold growth and "chalk molds"
0.90–0.95	Moist cakes, eg, carrot cake, some breads	Mold and yeast, bacterial spoilage, eg, "rope"
0.8–0.89	Plain cakes	Molds and yeasts
0.7–0.79	Fruited cakes	Xerophilic molds and osmophilic yeasts
0.6–0.69	Some dried fruits or fruited cakes	Specialized xerophilic molds and osmophilic yeasts
<0.60	Biscuits, chocolate, some dried fruits	No microbiological spoilage

Water activity can be used to predict the likely shelf life of a product. In the 1960s, Seiler[160] demonstrated that the shelf life of cake increases logarithmically as the a_w decreases (Figure 28-7). This relationship is widely used in the baking industry to estimate the mold-free shelf life of new products, reducing the need for extended storage trials. It is also used during new product development to identify the a_w needed to achieve the desired mold-free shelf life.

The rate at which yeasts spoil flour confectionery is also influenced by a_w. Fermentative spoilage problems are less common than mold spoilage, but at a given a_w fermentation tends to occur more quickly than mold spoilage. Since the materials that are most susceptible to fermentative spoilage, such as jams and icings, are used as fillings and coatings, this is very important because moisture migration from the product crumb to the filling can increase its a_w and reduce its expected fermentation-free life.

Product acidity can influence the rate of microbial growth, however, flour confectionery products are primarily spoiled by molds or yeasts, which tolerate a wide pH range. In these products, pH becomes most important because of its influence on the activity of any preservatives that may be included.

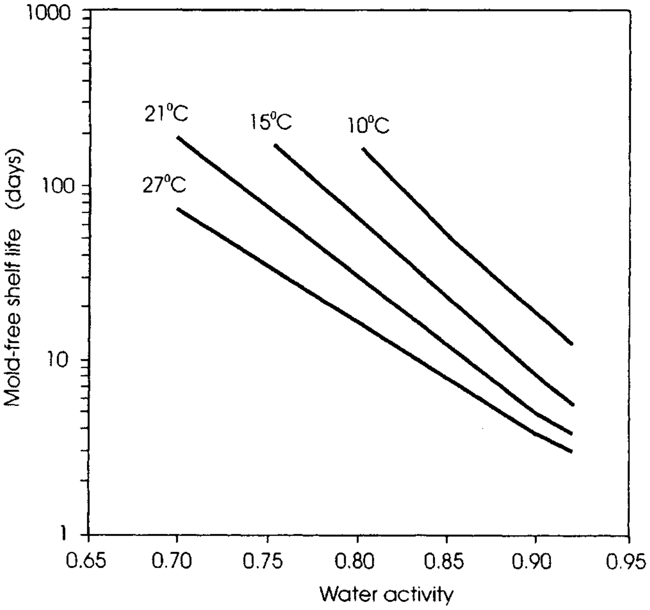


Figure 28-7 The Relationship between the a_w of a Cake and Its Mold-Free Shelf Life at Different Temperatures. The a_w was determined by the proportion of sugars, glycerol, salt, and water (from all sources including other ingredients) in the recipe. *Source:* Data from D.A.L. Seiler, Microbiological Spoilage of Bakery Products, *Swiss Food,* Vol. 3, pp. 42–44, © 1981.

Preservation Methods. The preservatives used most commonly for flour confectionery products are propionic acid and sorbic acid as well as certain of their salts. The maximum concentration of these preservatives permitted in the UK have recently been increased to 0.2% w/w of finished product (calculated as the acid)[5] for most products in which they are permitted. Since both are organic acids (or their salts), their antimicrobial action depends largely on the concentration of undissociated acid (or salt) present. The percentage of undissociated acid increases as the pH decreases; therefore the effect of these preservatives will depend on the pH of the product (Figure 28-8). Dramatic increases in mold-free life are theoretically possible, particularly in products with low a_w. However, high concentrations of preservative can cause off-odors and flavors within the product. It is difficult to generalize about the concentration of sorbate which a consumer might notice because of the possible masking effects of other flavors in the product. A level calculated to give a 50% increase in mold-free shelf life rarely causes such problems and the maximum permitted

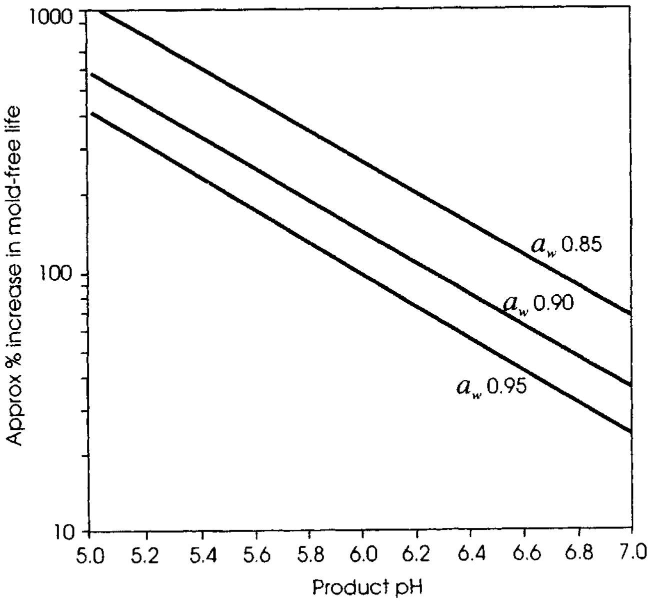


Figure 28-8 Approximate % Increase in the Mold-Free Shelf Life of Cake with Different pH Values Treated with Sorbic Acid at 1000 mg kg^{-1} Compared with a Control Cake of the Same a_w Containing No Sorbic Acid, pH Not Stated. *Source:* Data from D.A.L. Seiler, The Microbial Content of Wheat and Flour, *FMBRA Bulletin,* pp. 26–36, February 1978, © 1978, Campden and Chorleywood Food Research Association.

concentration of 0.2% sorbic acid may prove acceptable even when this will give a much greater than 50% increase in mold-free life. Nevertheless, sensory evaluation of a test batch of product is always recommended.

Modified atmosphere packaging using various gas mixtures has been shown to extend the shelf life of flour confectionery products. Use of 100% carbon dioxide around products with a_w below 0.90 has increased the mold-free life to up to five times that in air packs provided that loss of gas is prevented (Figure 28-9).[170]

28.5.4 Pastas

Pastas are prepared from durum wheat semolina (coarse particles of endosperm) or flour mixed with water into a very stiff dough and extruded through dies to give the final product shape. The dough contains around 30% moisture and may contain egg or other ingredients such as spinach, tomato, or other vegetables. On an industrial scale, pasta is usually mixed under vacuum in a continuous mixer, which may run for up to a week for nonegg pastas.[62]

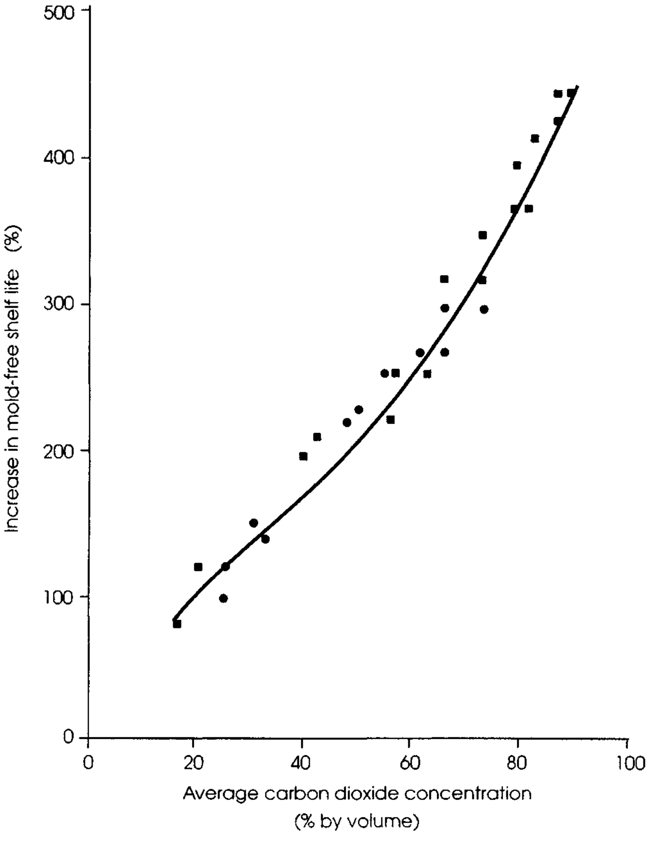


Figure 28-9 The Effect of Packaging in CO_2 on the Mold-Free Shelf Life of Cake. *Source:* Data from D.A.L. Seiler, Controlled Atmosphere Packaging for Preserving Bakery Products, *FMBRA Bulletin*, pp. 48–59, April 1984, © 1984, Campden and Chorleywood Food Research Association.

Dried Pastas

Most pasta is dried to a moisture content of 10% to 12%, which produces a product stable at ambient temperatures for very long periods. However, if dried pasta becomes moist, perhaps as a result of poor storage, it becomes susceptible to microbial spoilage. The initial microflora of dried pasta is variable, with recorded bacterial counts ranging between 10^3 g^{-1} and over 10^7 g^{-1},[40] and a range of molds including *Penicillium* and *Aspergillus* spp. are commonly present. Some pasta is sold fresh, sometimes filled with meat or cheese, and increasing amounts are sold from chilled display units after "pasteurization" and/or vacuum or modified atmosphere packaging.[54,190] Hence, there is potential for a wide range of microbiological problems.

The pathogen particularly associated with pasta is *Staphylococcus aureus*. Over 4% of Canadian-produced, dried, nonegg pastas, and over 5% of dried egg pastas, contained more than 25 *S. aureus* g^{-1}.[142] High concentrations of staphylococcal enterotoxin have been found in dried lasagna, which caused food poisoning in several European countries[56] and enterotoxin has been detected in dried pasta after storage for a year.[141] Clearly, it is essential to control *S. aureus* during pasta production and drying in order to protect the consumer. One of the difficulties to be overcome is that *S. aureus* is tolerant of lower a_w than other bacterial pathogens. It has been reported as multiplying in drying pasta until the a_w was below 0.86.[194] Therefore, drying needs to take place as quickly as possible consistent with avoiding stress-cracking of the product. Even when drying at the relatively low temperatures of 35° or 40°C, *S. aureus* did not produce enterotoxin in inoculated pasta when initial levels were 10^5 g^{-1} or lower[125] so the hygiene of the process prior to drying is clearly an important contributor to product safety.

Salmonella species derived from egg have been recorded as surviving the drying process for pasta and then remaining viable for several months in the final product.[40] *Salmonella typhimurium* and *S. infantis* inoculated into pasta before drying were still detectable after a year.[141] These organisms would be killed when the pasta was cooked, but most consumers would not consider dried pasta to be a risky material and are unlikely to handle it in such a way as to prevent cross-contamination to other foods. Therefore, there is a considerable responsibility on the manufacturer to ensure that *Salmonella* does not gain access to the product, in particular by using only pasteurized egg in pasta doughs.

Clostridium perfringens has also been reported in dried pasta.[40] However, pasta is unlikely to be a significant source of this organism under normal circumstances.

Fresh Pastas

Staphylococcus aureus can be even more of a problem in "fresh" (not dried) pastas than it is in dried. The a_w of fresh pasta is high (0.92–0.99), the higher values being associated with pastas filled with meat or cheese, eg, ravioli.[54] Trovatelli et al.[190] found more than 500 *S. aureus* g^{-1} in over 25% of factory-made pastas in the Bologna region of Italy with 14.3% containing over 10^4 g^{-1}. A similar proportion of pastas from small producers contained over 500 g^{-1}, but only 7.7% contained over 10^4 g^{-1}.[190] In another study, 12% of fresh pastas contained 10^2 to 10^5 *S. aureus* g^{-1}.[130] During storage at 5°C, the numbers declined over 4 weeks and no enterotoxin was detected, but after 4 weeks at 16°C, 22% of the samples contained 10^3–10^7 *S. aureus* g^{-1}, and 8% contained enterotoxin. This demonstrates quite clearly the importance of good cold storage during distribution and display of fresh pastas.

Other microbes are found in fresh pastas, potentially in large numbers. In a study of industrial nonegg pastas, nearly 70% of the samples of simple pastas (tagliatelle, spaghetti, and fettucini) tested gave aerobic plate counts $>10^6$ g^{-1} (the Italian legal maximum).[106] An even higher proportion (87%) of samples of filled pastas had aerobic plate counts $>10^6$ g^{-1}. In contrast, just under 30% of samples of egg pasta from small manufacturers had total viable counts of 10^6 g^{-1} or more.[104] Fecal coliforms in nonegg pastas were detected at a level greater than 10^3 g^{-1} in 17% of filled and 3% of simple pastas.[106] An even higher proportion of fresh pastas produced by small manufacturers contained $>10^3$ fecal coliforms g^{-1} (5% to 20% between 1984 and 1986[135]). Bacterial species identified among coliforms isolated from pastas include *Klebsiella oxytoca, Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter agglomerans, Escherichia coli, Serratia* spp., *Providencia alcalifaciens,* and *Hafnia alvei.*[48]

"Long-Life" Pastas

Fresh pastas are increasingly being packed under vacuum or modified atmospheres to extend the shelf life of the product and provide the consumer with a convenient, attractive product. These products are often distributed in chill conditions. The technologies can certainly delay the onset of mold spoilage.[32] Fresh pastas packed in air and stored at 4°C showed visible mold growth after 6 to 7 days, whereas in vacuum packaging no mold growth occurred within 14 days. Bacteriological spoilage may occur, however, particularly under temperature-abuse conditions. One curious manifestation of bacteriological spoilage was a pink discoloration of vacuum packaged gnocchi held at 8°C caused by growth of *Bacillus polymyxa.*[180]

High aerobic bacterial counts (10^6 to 10^7 g^{-1}) have been detected in vacuum packed pastas.[8] In the same study, aerobic mesophilic spore counts of 10^3 to 10^4 g^{-1} were obtained but no *S. aureus* was detected. In contrast, in a Canadian study, 17.5% of plain and 33.7% of filled pastas in modified atmosphere packs contained detectable levels of *S. aureus.*[42]

In an investigation of the ability of proteolytic strains of *Clostridium botulinum* to multiply and form toxin in pasta, 6.6×10^2 spores g^{-1} were inoculated into pastas with a range of a_w, which were then packed in atmospheres of N_2 or N_2 and CO_2.[54] Toxin was formed in products with an a_w higher than 0.95 stored at 30°C. Most of the flat noodles were at an a_w below the limit that allowed growth of proteolytic *C. botulinum*, but most of the filled pastas had an a_w higher than 0.95. Admittedly, this study was under conditions of severe temperature abuse but it illustrates well the need to understand the effect of process or packaging changes on the safety of traditional products.

Cooked Pastas

S. aureus can grow well on cooked pasta. For example, in cooked egg pasta inoculated with approximately 100 *S. aureus* g^{-1}, production of staphylococcal enterotoxin A occurred in 3 days at 15°C and only 1 day at 25°C.[56] Hence, cooked pasta needs to be treated with some care to minimize the opportunities for contamination with *S. aureus* and should be maintained in chilled conditions to retard its growth. Holding hot (>63°C) prior to serving would be an alternative in a catering situation.

28.6 CONCLUDING REMARKS

Cereals and cereal products provide the staple food source for a large proportion of the world's population. Cereals are relatively cheap, nutritious, palatable, and versatile foodstuffs. Traditional technologies for storing and processing cereals give products with a record so good, overall, that their safety is taken for granted by the consumer. This compares favorably with other food groups, particularly meat, poultry, eggs, and dairy products: all perishable foods that readily support microbial growth and have a history of causing food poisoning when not stored or prepared correctly. It would be unwise, however, to assume that there are no microbiological hazards associated with cereal-based foods. Those currently known or suspected have been reviewed in this chapter but there is much still to discover.

Little is known about the bacteriology of cereals relative to the wealth of literature relating to fungi and mycotoxins. The range of bacteria, including pathogens, spoilage organisms, and commensals typically associated with different cereals, is not well documented. The source of pathogens found in cereals has not been rigorously identified and the role (if any) of cereal meals and flours as vehicles for the spread of pathogens is not well understood. With the wider implementation of HACCP systems in the food industry, long-standing assumptions about cereal products are being questioned and the answers are not always available.

"Industrial" bread baking as practiced in the UK, US, and other developed countries has been honed to very high levels of efficiency with the emphasis on cost and product appearance. More consideration is now being given to flavor, and the use of mixed sourdoughs, modified yeast cultures, and fermentation technology to this end is beginning to be explored.

Cereal products similar to traditional items but intended to have longer life (and therefore be more convenient) have been developed over several years. Over the same period, products with "moister eating" character and fewer preservatives have been produced. Both developments have made use of technologies not traditionally associated with cereal foods, for example, modified atmosphere packaging and chilled storage. There are two challenges here. The first is to identify correctly the factors that govern the safety of the new products and the second is to educate consumers to recognize the different handling and storage conditions that may be required. There remain plenty of questions for cereal microbiologists to explore, and the activities of the food technologists are likely to generate more questions, not fewer, in the future.

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29 Yellow Fat Products (Butter, Margarine, Dairy and Nondairy Spreads)

Maddy M. van Zijl and Pieter M. Klapwijk

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29.1 INTRODUCTION

Yellow fat products are used for many purposes including spreading on bread, cooking and baking, and as topping on meals or meal components. Classical examples are butter and margarine; water-in-oil emulsions that contain at least 80% fat (w/w). Nutritional recommendations to limit the amount of fat, in particular saturated fats, in the diet have resulted in a decline of the consumption of yellow fat spreads in developed countries and a relative shift to low-fat products. The consumption of butter and related products seems to have stabilized, whereas the consumption of margarines and related products is declining.[36,46]

Butter is an ancient dairy food in which the milk fat has been concentrated. Salt and starter cultures may be added. Butter was originally made directly from milk on a small scale. Its factory production dates from about 1850.[78] Most changes in butter manufacture have concerned the process design, and new types of butter-making processes have been introduced.[33] The composition of butter is subject to stringent regulations and thus has not changed much over the years. Low-fat variants of butter have recently appeared on the market, together with products based on mixtures of butter/butter fat and vegetable fat blends (melanges),[52] which may be produced by a typical butter and/or margarine process and can be either full-fat, medium-fat, or low-fat.

Margarines and related products are made of water, oil, and fat to which ingredients such as milk or milk products, salt, and thickeners may be added depending on the type of product. Margarine was developed during the 19th century as an alternative to butter, which at the time was in very limited supply. The oils and fats used for margarine manufacturing have undergone major changes since the production of the first margarine in 1869. Animal fats, such as lard, tallow, and fish oil, were used in margarine production until the early 20th century when they were replaced by vegetable oils and fats, eg, coconut oil, cottonseed oil, and, more recently, by soya oil and sunflower oil. Fat blends are designed so that they will meet requirements such as spreadability at refrigerator temperature, cooking or baking performance, and keepability. Since the mid-1960s, several margarines have been developed based on fat blends high in polyunsaturated acids. Another important trend is the development of products with a low fat content for spreading on bread. The first product containing 40% fat was introduced in 1964 in the United States (US), and the first product containing 20% fat in 1988 in the United Kingdom (UK). Products with fat levels from 20% to 80% have now gained widespread acceptance.[42,51,52,56,57] Recently, products with fat levels as low as 3% have successfully been introduced in the US, UK, and The Netherlands. In addition, products have been developed for specific purposes, eg, liquid, squeezable margarines for cooking and baking.

Yellow fat products manufactured in developed countries are generally of a very good microbiological quality. Fat can act as a barrier to microbial growth, and for this reason systems with a continuous fat phase are usually much more stable than systems with a continuous aqueous phase. The decrease in fat content in many products can have consequences for the microbiological stability of these products, as they often consist of a water-in-oil emulsion with coarser water droplet sizes. In addition, a higher water content results in lower levels of salt in the aqueous phase. There are, however, no indications that industrially produced yellow fat products play a significant role in foodborne disease, despite the recent case of contamination of butter with *Listeria monocytogenes*.[50] This is probably due to a continually increased attention to hygiene during manufacturing,[19,35,48] to the quality of raw materials, and to the pasteurization conditions applied, which ensure absence of vegetative pathogens. It remains necessary, however, to pay considerable attention to the microbiology of yellow fat spreads, especially of new products and processes.[44]

29.2 BUTTER

29.2.1 Composition and Structure

Composition

The composition of butter is strictly regulated in many countries. Butter consists of at least 80% milk fat, a small amount of nonfat milk solids, salt (sodium chloride if any), and water. A lactic starter culture, colors, and neutralizing agents may be used during manufacture. It is normally not permitted to add preservatives, other than salt, or anti-oxidants to butter.[9,59] In Western Europe butter and butter-like products are described as: "Products in the form of a solid malleable emulsion, principally of the water-in-oil type, derived exclusively from milk and/or certain milk products, for which the fat is the essential constituent of value. However, other substances necessary for their manufacture may be added, provided those substances are not used for the purpose of replacing, either in whole or in part, any milk constituents."[17] Butter is more specifically defined as: "The product with a milk fat content of not less than 80% but less than 90%, a maximum water content of 16% and a maximum dry non-fat milk-material content of 2%."[17]

Most countries in Europe favor cultured butter, which is unsalted, or at most salted to a level not exceeding 0.5% (w/w). In the English-speaking countries of the world (eg, UK, US, and Australia)[34,59] and in Japan and India[34] sweet cream butter is sold almost exclusively, often with 1.5% to 2% (w/w) salt. Finally, unsalted sweet cream butter is preferred in Czechnia and Slovakia.[34] Cultured butter contains lactic acid in place of lactose and usually has, if not neutralized, a pH of 4.6–5.3. In microbiological terms, the aqueous phase of sweet, unsalted butter is much more unstable than that of salted or cultured butter.

Structure

Butter is, like margarine, a fat-continuous (water-in-oil) emulsion in which the water is compartmentalized in small droplets. The microbial stability of butter depends largely on a proper water dispersion. If the water is dispersed in small droplets, outgrowth of microorganisms will be limited and die-off may occur during storage. When the droplets are too coarse or when water channels are present, the aqueous phase composition, ie, the pH and salt-in-water concentration, becomes more important for the keeping quality.[39] This was recognized many years ago and microscopical methods were developed to characterize the water dispersion in butter.[58,64] Different processing regimes may result in different microstructures. Electron-microscopical examinations demonstrate that in properly worked butter, the aqueous phase is almost exclusively present as isolated, globular or elongated droplets with diameters up to about 30 μm, covered with high-melting butter fat crystals.[10,62] Water channels are usually not present in properly worked butter.

29.2.2 Initial Microflora

The initial microflora of a product is determined by the microbiological quality of its raw materials, ie, its ingredients and packing materials. The primary ingredient for butter is cream, and the initial microflora of butter is therefore to a large extent derived from the cream.

Cream

In the modern dairy situation, milk is collected on farms in refrigerated bulk tanks and transported to the dairy factory, where the fat in the milk is concentrated to obtain cream with 38% to 42% fat.[33] The cream may also be separated at the farm, which often results in cream of a lower microbiological quality, as it may be held for a week under poor hygienic conditions and not always under refrigeration.[9] Under these conditions, souring may occur spontaneously, eg, by growth of *Lactococcus lactis*, and undesirable microorganisms will start to grow. Growth of yeasts and molds, eg, *Geotrichum candidum*, may be abundant.[24] This situation is likely to occur nowadays only in areas with less advanced dairy industries.

Raw cream should be processed immediately to prevent deterioration. During storage of nonpasteurized milk or cream, psychrotrophic Gram-negative bacteria such as *Pseudomonas* spp. and *Flavobacterium* spp. may grow, leading to proteolytic and lipolytic changes and to off-flavors.[12,24,40] Off-flavors or microbial exolipases present in the cream may be carried over to the finished product and affect its quality. Vegetative pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157, *Campylobacter*, and *Salmonella* spp. may be present in the milk. To kill all the relevant microorganisms, the milk and/or the cream is normally pasteurized before butter manufacture (see also Section 29.2.3). Suggested microbiological criteria for cream are aerobic plate count of <1000 cfu ml^{-1} and yeast, mold, and coliform count of <1 cfu ml^{-1}.[59] *Bacillus cereus* should be monitored regularly in view of increasing occurrence in milk of *B. cereus*,[27] spores of which survive pasteurization of milk and cream and can germinate and grow, although very slowly, at low temperatures. *B. cereus* may be relevant for sweet, unsalted, or low-salted products with coarse water droplets.

Other Ingredients

Cream may be fermented by the use of starter cultures consisting of lactococci and/or leuconostocs (cultured, soured, or ripened cream). Starter cultures can be a source of contamination and care should be taken to keep them free of contaminants. Water must always be of potable quality and drawn from a reliable source. When water is used in butter manufacture after pasteurization, eg, for washing, the water should be of a very good, potable quality. Other ingredients such as salt, the coloring agent beta-carotene, and neutralizers such as sodium carbonate are generally free of microbial contamination because of the way they are manufactured. The chemicals should be of food-grade quality.

Packing Materials

The packing material should be of a good microbiological quality. Of specific importance is the level of contamination with molds (spores), which should be as low as possible because their presence can result in mold spoilage at the surface of butter when free moisture is available at the surface.

29.2.3 Processing and Its Effects on the Microflora

The butter process (Figure 29-1) starts with sweet or soured (cultured) pasteurized cream.[33] Biological fermentation of the cream produces a pleasantly aromatic flavor in the butter. In the past, cream was mostly prepared on the dairy farms where butter was also prepared. Today, the manufacturing of cream is usually separate from that of butter. The butter process can be divided roughly into two stages: (1) a churning stage in which the fat in the cream is concentrated to about 80% after which buttermilk is drained off, and (2) a working stage, in which the concentrated cream is worked into butter. In between, ingredients such as salt, colors, and concentrated starter cultures may be added. The manufacturing of cream and butter are described in more detail in the next sections.

Cream Manufacture

The milk, after reception at the factory, is usually chilled and held at about 5°C before it is separated at 46° to 49°C to yield cream with a fat content of 38% to 42%.[33,59] In some plants, the milk is pasteurized first and then cooled to separation temperature. In others, the milk is heated to separation temperature, and the cream is pasteurized subsequently. It is usual to pasteurize the cream after separation, even when the original milk is pasteurized.[59] Heat treatments (plate heat exchanger) involve temperatures in the range of 85° to 112°C,[33] but 85° to 95°C for 10 to 30 seconds is most common.[78] Batch pasteurization at longer holding times is now obsolete or confined to low technology plants in some areas of the world.[9]

If the cream is not to be soured, it is cooled quickly to 3° to 5°C after pasteurization and held at this

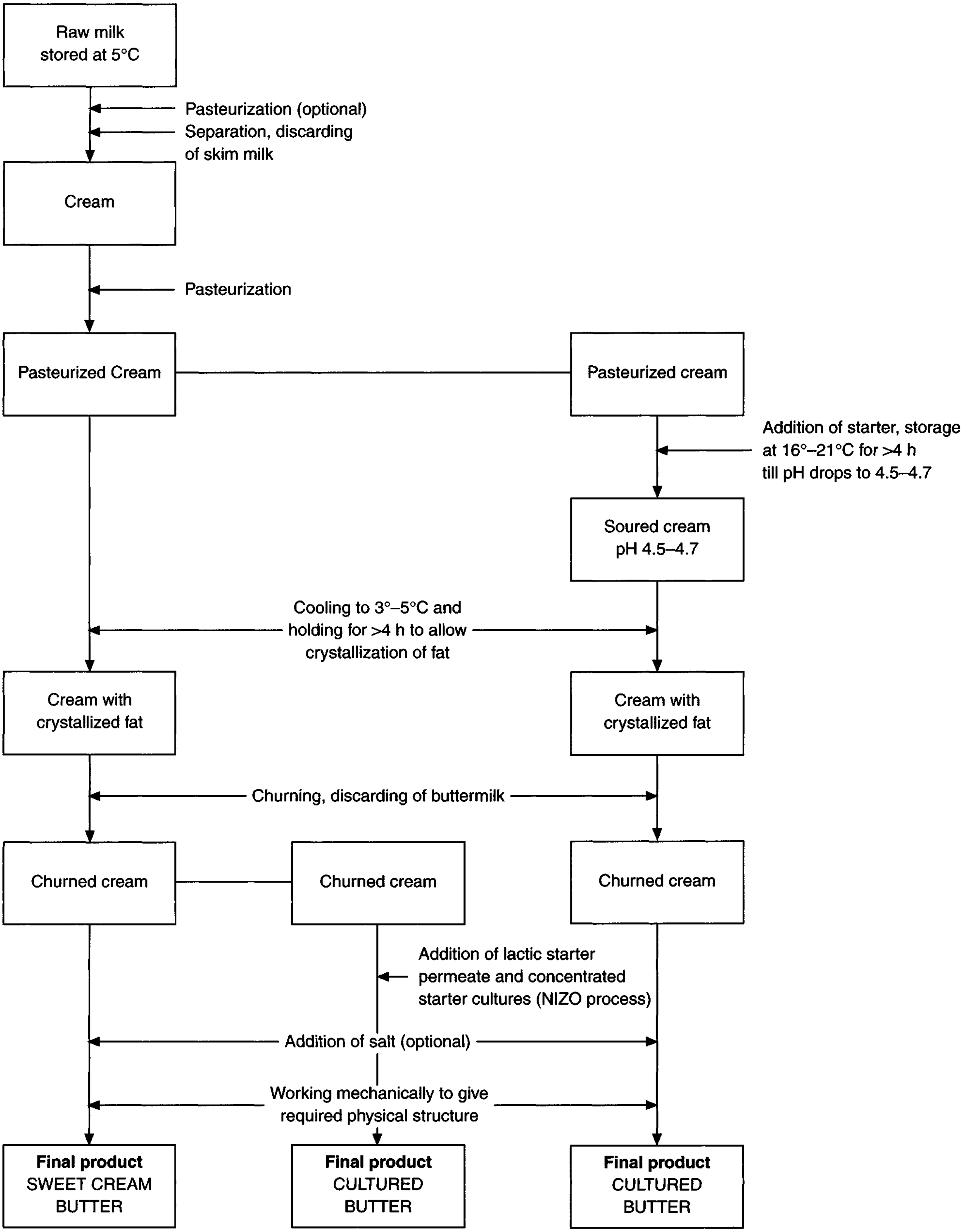
Raw milk stored at 5°C
Pasteurization (optional)
Separation, discarding of skim milk
Cream
Pasteurization
Pasteurized Cream
Pasteurized cream
Addition of starter, storage at 16°–21°C for >4 h till pH drops to 4.5–4.7
Soured cream pH 4.5–4.7
Cooling to 3°–5°C and holding for >4 h to allow crystallization of fat
Cream with crystallized fat
Cream with crystallized fat
Churning, discarding of buttermilk
Churned cream
Churned cream
Churned cream
Addition of lactic starter permeate and concentrated starter cultures (NIZO process)
Addition of salt (optional)
Working mechanically to give required physical structure
Final product SWEET CREAM BUTTER
Final product CULTURED BUTTER
Final product CULTURED BUTTER

Figure 29-1 Diagram of the Butter Production Process

temperature for at least 4 hours for ageing (crystallization of the fat phase). When it is to be soured, the cream is cooled to 16° to 21°C, fermented with a starter culture containing acid producers (*Lactococcus lactis* subsp. *lactis* and/or *cremoris*), and diacetyl producers (*Leuconostoc mesenteroides* subsp. *cremoris* and/or *dextranicum* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*) for 4 to 6 hours (or longer) until the pH drops to 4.5 to 4.7, and further cooled down to churning temperature.[33,59] The soured butter will be particularly sensitive to oxidation if the product is salted to levels higher than 0.5%, because of the combined effect of low pH and the catalytic action of the salt.[59] In some countries, soured cream is neutralized to pH 6.0 by the addition of alkaline salts such as calcium oxide, sodium carbonate, or sodium hydroxide (continuous process).

Pasteurization of the cream is designed to destroy relevant vegetative microorganisms such as *Pseudomonas* spp. and *Flavobacterium* spp. and pathogens, such as *Listeria monocytogenes*, *E. coli* O157, *Campylobacter* spp., and *Salmonella* spp. Bacterial spore-formers and, depending on the temperature applied, some of the more heat-resistant vegetative spoilage flora, such as streptococci and *Microbacterium lacticum*, will not be killed.[28] Another important objective of the heat treatment is to improve the keeping quality by the destruction of enzymes, in particular lipases inherent in the raw cream or of microbial origin, reduction of the intensity of any undesirable flavors, and, at temperatures >105°C, production of anti-oxidant sulphydryl groups. The migration of the pro-oxidant copper from the serum into the fat phase may, however, have an opposite effect. Finally, pasteurization will completely liquify the fat for subsequent control of crystallization.[33,78]

Growth of microorganisms in the milk or cream should always be kept to a minimum by controlling time and temperature of storage. When high numbers of microorganisms are present as a result of abundant growth before pasteurization, not all relevant microorganisms may be inactivated by the usual pasteurization conditions. Bacterial spores are not inactivated by pasteurization and can germinate and grow when milk or cream of neutral pH is not sufficiently cooled. Pasteurization is a critical control point, because there are no further decontamination treatments in butter manufacture. Any failure in pasteurization could lead to the survival of pathogens. Subsequent process steps should be designed such that multiplication of those microorganisms surviving pasteurization is prevented and recontamination is minimized.

Butter Manufacture

In all major butter-producing countries, butter manufacture is nowadays a continuous process.[9,33,34,59,78] The technology is quite advanced and cleanable-in-place, stainless steel equipment is common.[35] The process starts with churning of the cream. By intensive agitation at 5° to 7°C the fat globules in the cream are converted to fat granules. The granules are then separated from the other constituents of the cream, leaving buttermilk. This results in a two-fold concentration of the fat in the cream. During churning most microorganisms are retained in the buttermilk. Cream for butter making may be washed after churning, if it is obtained from poor-quality milk, to remove off-flavors; however, this may result in recontamination of the butter with microorganisms, eg, *Pseudomonas* spp. With improved methods of manufacture and hygiene, it is now usually considered unnecessary to wash the butter granules after churning, thereby eliminating this potential source of contamination. Butter made without washing is also considered to be of better flavor.[78] In continuous butter manufacture, washing is normally not possible.

If salt is added, this is done after the last point from which buttermilk drainage can occur, to prevent entry of salt into the buttermilk. A concentration of 2% salt in the product will result in a concentration of 11% in the aqueous phase if the water content is 16% [salt/(salt + water)]. As a result, the salt-in-water concentration is theoretically increased to a range that inhibits many microorganisms. However, the salt is not always uniformly distributed over the compartmentalized aqueous phase. The butter reaching the salting stage will contain up to 14% water, which means that in order to obtain 2% of salt in the product and maintain the legal limit of 16% water content, the salt must be added as a 50/50 salt-in-water slurry. Consequently, the salt may not always be uniformly distributed over the product. This explains the occurrence of microbial deterioration even in highly salted butter.

Cultured butter has traditionally been made from cream soured by starter cultures. The sour buttermilk resulting from this process has little market value, in contrast to sweet buttermilk. For this reason increasing use is being made of the so-called NIZO process, which starts with sweet cream and yields sweet buttermilk. The NIZO process was developed by the Netherlands Institute for Dairy Research (NIZO). In this process, after churning, a lactic starter permeate that contains a high concentration of lactic acid and one or two aromatic starter cultures rich in diacetyl is worked directly into the butter to reduce the pH to <5.3 and to add flavor to the butter.[33,79] The lactic starter permeate is prepared by souring pasteurized whey with a reduced lactose content, or a similar substrate containing only milk components, by a lactic acid bacterium, eg, *Lactobacillus helveticus*, which is able to produce considerable lactic acid even below a pH of 4.5. Then the culture is submitted to ultrafiltration to remove proteins and bacteria, and the permeate is concentrated by evaporation to contain 11% to 12% lactic acid (pH 3.0). Well-known aromatic starter cultures are the culture of starter 4/25 containing *L. lactis* subsp. *lactis* biovar. *diacetylactis* as a strongly aromatic bacterium and starter Fr19 containing *Leuconostoc mesenteroides* subsp. *cremoris* in skim milk (pH 4.7).[79] The lactic starter permeate and starter culture 4/25 are mixed and aerated before being worked into the butter. The second starter culture, Fr19, is added separately. Recently, it was demonstrated that the starter culture of 4/25 can be substituted by a distillate of that culture, leaving only one starter to be cultured.[80] Because of the good taste and keeping quality of the butter, the NIZO process is widely accepted.[33] The use of a permeate or distillate of a starter culture may further add to the microbial quality of butter, as fermentation is a vulnerable step.

After churning, the butter is worked mechanically to obtain the right physical structure. A major process aim is to obtain small water droplets. Both underworking and overworking produce a final butter with coarse water droplet size distributions or even free moisture. Water droplets should preferably not exceed 10 µm in diameter. As droplet size distributions are skewed with a tail of larger droplets, this means that approximately 50% of the total water volume should be in droplets with diameters smaller than about 3 µm (volume-weighted geometric mean diameter, $\bar{D}_{3,3}$ in µm), while at the same time less than 5% of the water volume will be in droplets with diameters >10 µm.

Butter may be packed in bulk, usually up to 25-kg units, or in consumer-size portions of 10 to 500 g. Traditionally, parchment was used as the primary packing material and is still widely used, especially for larger units, but other materials have become available.[34] Larger units may, in addition to wrappers of parchment, be packed in vinyl bags. Consumer-size products are often packed in wrappers of aluminum foil laminated on the inside with parchment by means of wax, in wrappers of polyethylene, or in plastic tubs. A major disadvantage of parchment as primary packing material is, from a microbiological point of view, that cellulolytic molds are able to grow on wet parchment. To prevent mold growth, the parchment is sometimes soaked in concentrated brine plus 0.5% sorbic acid,[59,78] but this is not allowed in many countries. In general, however, to prevent mold spoilage the butter must have a fine water distribution and a surface without free moisture, and mold contamination by the environment or the packing material should be avoided.

Hygiene of Production

Butter is a rather vulnerable product and therefore strict hygienic measures should be applied. Most modern production lines are closed after pasteurization, thereby excluding contamination from the environment after pasteurization. Hygienic equipment and adequate maintenance procedures are essential to prevent contamination.[33] It is furthermore of utmost importance that the equipment is properly cleaned and disinfected before start-up of the process. The product is exposed to the environment during packaging, at which stage contamination may occur. The air in the packing environment should be of a very good quality, as butter is vulnerable to mold growth. Cardboard secondary packing material may be an important source of mold spores, especially if recycled cardboard is being used. To prevent contamination, the cardboard packing material should not be handled in the packing environment, but in a separate room. Free moisture will trigger the growth of mold spores. Moisture may condense on the surface of the product if it is moved from a cool area to an area of high humidity at the time when it is being wrapped and labeled. It is important, therefore, to control the humidity in the environment. Butter packed in bulk may be repacked in smaller packs later on. Repacking may result in enlargement of the water droplets in the butter, making the product less stable from a microbiological point of view. Butter that is repacked should be fresh or of extremely good microbiological quality, because repacking can cause the spread of microorganisms throughout the production batch. Frozen butter must be thawed in a dry and well-ventilated room to prevent moisture condensation on the butter.

Closed Shelf Life and Consumer Use

Bulk-packed butter may be stored for long periods at very low temperatures, eg, for 6 months at –15°C or a year at –30°C, which prevents growth of microorganisms. Butter should always be stored at chill temperatures (≤10°C). The storage time is then generally limited to 6 to 12 weeks. Cool and dry storage and transport is necessary to prevent mold spoilage. There is an increased risk of mold growth during cooling, freezing, and thawing when moisture can accumulate between the primary packing material and the butter surface. Because of the continuous fat phase of butter, growth of microorganisms will be localized, as is explained in Section 29.5. In addition, depending on the composition of the aqueous phase many microorganisms will not grow under refrigerated conditions. If butter is whipped or spiced, precautions should be taken to ensure that pathogens are not introduced during this process.

29.2.4 Spoilage Associations and Problems Due to Pathogens

Spoilage Associations

Butter manufacture is a fairly sensitive process from a microbiological point of view and, therefore, microbial spoilage attained considerable attention in the past.[24,28,68] With improved methods of production and hygiene, microbial spoilage of butter, produced in a modern dairy operation, is uncommon. Defects of nonmicrobial origin, such as oxidation of fat at the surface and rancidity resulting from fat hydrolysis, are probably the most frequent defects of butter nowadays. Nevertheless, microbial spoilage still occurs occasionally. Typical butter defects of microbial origin are described below.

Growth of *Alteromonas putrefaciens* (previously called *P. putrefaciens*) may lead to surface taint.[84] The surface taint defect has also been attributed to *Flavobacterium* species,[24,40] eg, to *Flavobacterium maloloris.*[24] The defect initially involves the surface layers of butter but the characteristic odor and taste may develop quickly throughout the product. It involves decomposition of the protein portion of the product and is manifested by a putrid, decomposed, or cheesy flavor, apparently from isovaleric acid or a closely related compound.[15] *Pseudomonas fragi* and *Pseudomonas fluorescens* are associated with the formation of fruity odor, caused mostly by ester formation. Because both species are lipolytic as well as proteolytic, they are also responsible for rancid odors.[24] Growth of *Pseudomonas nigrifaciens* may cause a black discoloration.[24] A variant of *L. lactis* (formerly *S. lactis* var. *maltigenes*) may cause the so-called malty-flavor defect related to the formation of 3-methylbutanal.[37] If the organism grows extensively in the cream before pasteurization, the flavor can carry over to the pasteurized cream.

Molds are still important spoilage microorganisms of butter and can result in surface discoloration and off-flavor. A variety of genera have been implicated such as *Penicillium*,[45,78] *Oospora*,[45] *Mucor*,[45] *Geotrichum*,[78] *Aspergillus*,[78] and *Cladosporium*.[45,78] Yeasts are potentially important spoilage microorganisms. Some species are lipolytic and can grow in the presence of high concentrations of salt, at low pH, and at low temperatures; *Rhodotorula* spp., *Saccharomycopsis lipolytica*, *Cryptococcus laurentii*, and *Candida diffluens* were reported as predominant yeasts in retail samples of butter.[21] Yeasts that have been implicated in spoilage include *Candida lipolytica*, *Torulopsis*, *Cryptococcus*, and *Rhodotorula*.[78]

Rancid flavor comes mainly from free butyric acid that arises from the hydrolysis of butter fat. This reaction may be catalyzed by naturally occurring lipase in the milk or by enzymes secreted by microorganisms. Milk lipase is destroyed by pasteurization.[14] Most, but not necessarily all, lipases of microbial origin will be fully inactivated by pasteurization. Microbial lipases can be rather heat resistant and their inactivation may depend on the pasteurization conditions applied. The potential carry-over of residual enzyme activity, bacterial toxins, and off-flavor to the pasteurized cream are major reasons to strictly limit microbial growth before pasteurization, even though the previously mentioned microorganisms will all be destroyed by proper pasteurization.

Problems Due to Pathogens

There are a few cases known of foodborne illness caused by butter. The reported cases concern butter and whipped butter (three cases) contaminated with *Staphylococcus aureus*,[3,74,75] blended margarine and butter products contaminated with *S. intermedius*,[43,77] green butter (made by adding parsley to butter) contaminated with *Citrobacter freundii*,[69] garlic butter contaminated with *Campylobacter jejuni*,[6] and butter contaminated with *Listeria monocytogenes*.[50] Another recall of butter, probably unsalted or lightly salted, contaminated with *L. monocytogenes* was also reported, but not related to foodborne illness.[5] The reported cases will be discussed in more detail below.

In 1970, butter was recalled by a butter manufacturer in the US in a multistate area, because the butter contained excessive numbers of bacteria and there were suspicions of a staphylococcal contamination.[3] Illness had been reported by consumers after consumption of the product. In the same year, food poisoning by consumption of whipped butter contaminated by *S. aureus* at a restaurant was also reported.[74] The contamination was traced to the butter used to prepare the whipped butter. *S. aureus* could not be detected in the milk that was added to the butter to prepare the whipped butter. The same brand of butter was implicated as the cause of a single case of typical staphylococcal food poisoning in the same year. This butter was shown to contain staphylococcal enterotoxin A.[74] In 1977, whipped butter produced by a a single manufacturing plant in the US was implicated in a multistate outbreak of presumed staphyloccocal food poisoning, which involved over 100 people.[75] Up to 10^7 *S. aureus* g^{-1} were isolated from lots of the whipped butter involved. Whipping of butter increases the risk of *S. aureus* growth, because water or milk may be added to the butter before whipping. Therefore, the product should be subjected to strict hygiene, refrigerated storage (≤7°C), and a restricted shelf life. The toxin of *S. aureus* is very heat stable and once formed as a result of poor sanitary conditions in the prepasteurization stage, might be carried over to the pasteurized product.[45]

Food poisoning by *S. intermedius* in 1991, caused by consumption of contaminated, blended margarine and butter products, involved more than 265 people in the US.[4,43,77] A single strain was identified as the causative agent, which suggests a common source of contamination rather than post-process contamination of the food products (see also Section 29.3.4). A German outbreak of infection with *C. freundii*, probably in 1994, which resulted in severe gastroenteritis and haemolytic uraemic syndrome (HUS) at a nursery school and adjacent kindergarten, was caused by consumption of green butter contaminated with verocytotoxinogenic *C. freundii*.[69] Green butter was used to prepare sandwiches and was made with contaminated parsley from an organic garden in which pig manure was used as fertilizer. Genetically identical verocytotoxinogenic *C. freundii* was isolated from most patients and from the parsley of the organic garden. Unfortunately, the green butter itself was no longer available for examination. In view of the rapid onset of disease and the severity, *C. freundii* had probably multiplied in the butter before consumption. In addition, genetically heterogeneous, verocytotoxinogenic *Escherichia coli* O157 was isolated from some of the patients and from the parsley. The genetic heterogeneity of the *E. coli* isolates together with their scarcity suggested a marginal involvement of *E. coli* in the outbreak. An outbreak in 1995 of *Campylobacter* enteritis involved about 30 people who had eaten at a restaurant in Louisiana.[6] *C. jejuni* was isolated from the patients, and epidemiological studies revealed that the illness was associated with garlic butter prepared by the restaurant.

Infectious pathogens such as *Salmonella* and *L. monocytogenes* may occur in raw milk. These microorganisms will not survive proper pasteurization, therefore butter made from pasteurized milk/cream should not contain these organisms unless post-pasteurization contamination occurs. If *L. monocytogenes* is present in butter it can survive for months when frozen (–18°C).[60] There have been a few published reports on investigation of the presence of *L. monocytogenes*[5,29,50,53,54] and *Salmonella*[11] in butter in recent years and these bacteria were not detected in the samples studied, except for a recent incident of serious contamination with *L. monocytogenes* in Finland.[50] A recall was reported in 1994 in the US of unsalted and lightly salted butter contaminated with *L. monocytogenes*,[5,63] but no cases of food poisoning were linked to this contamination. In a case-control study of a cluster of perinatal listeriosis in California carried out over a period of 6 months in 1987–1988, butter was identified as a possible vehicle for infection,[13] but no direct evidence was available. However, in 1999 a serious outbreak of food poisoning implicating butter contaminated with *L. monocytogenes* was reported in Finland.[50] Eighteen people developed listeriosis between December 1998 and February 1999, four of whom died. The mean age of patients was 57 years (range 18 to 85); all had serious underlying illnesses and were undergoing treatment in several hospitals. Most of the disease-causing strains (14/18) were of a rare serotype, 3a. *Listeria monocytogenes* serotype 3 was isolated from several places in the production facility of a Finnish dairy plant, including environmental and butter samples from the dairy plant and butter samples from the cold store of the dairy; isolates were also obtained from the kitchen of a hospital. *Listeria monocytogenes* was detected in most of the 7 g and 10 g packages of butter tested, at levels of <100 cfu g^{-1}, and once at a level of 11,400 cfu g^{-1}. The isolates from butter and epidemic isolates were indistinguishable by pulsed-field gel electrophoresis (PFGE). This PFGE-type had not been isolated previously from any other foods in Finland. The Department of Infectious Disease Epidemiology of the National Public Health Institute subsequently conducted an epidemiological investigation to strengthen the evidence of an association between consumption of butter and illness.[50]

29.3 MARGARINE

29.3.1 Composition and Structure

Composition

The composition of a margarine is, in general, strictly regulated. The definition of a margarine in the US is: "Margarine (or oleomargarine) is the food in plastic form or liquid emulsion containing no less than 80 percent fat."[76] In Western Europe, yellow fat products other than butter and melanges are described as: "Products in the form of a solid malleable emulsion, principally of the water-in-oil type, derived from solid and/or liquid vegetable and/or animal fats suitable for human consumption, with a milk fat content of not more than 3% of the fat content." The category margarines is described as: "The product obtained from vegetable and/or animal fats with a fat content of not less than 80% but less than 90%."[17]

A wide range of oils and fats may be used, eg, soya oil, palm oil, and sunflower oil. In addition to the fat blend, water (16% to 18%) and emulsifiers are required. Other ingredients include salt (sodium chloride), milk or milk products (sweet or cultured skim milk powder, buttermilk powder, sweet or acidified whey powder, milk protein), acidulants such as citric and lactic acid, preservatives, vitamins A and D, flavors, and colors such as beta-carotene. The concentration of salt may vary from 0% to 2% (w/w) or up to 2.5% (w/w) in the finished product (calculated 0% to 11% w/w on aqueous phase). In some countries, such as the UK, high salt levels are preferred. For dietary reasons, salt may be omitted. Milk or milk products are generally added as a powder in concentrations of 0.1% to 1.0% of the product. Citric and lactic acid, separate or in combination, are often used to adjust the pH. Antimicrobial preservatives that may be added in the US include sorbic acid (maximum 0.1% of the product) and benzoic acid (maximum 0.1%), or a combination thereof (maximum in total 0.2%). In Western Europe, only sorbic acid or its salts is allowed at a maximum concentration of 0.1% of the product (acid concentration).[18] The pH of the aqueous phase of a margarine is usually between 3.5 and 6.0.

Structure

Margarine contains a continuous fat phase with a network of fat crystals, which gives the consistency to the product. Most of the water is normally present as finely dispersed droplets with a diameter of <10 μm, with 50% of the total water volume in droplets smaller than about 3 μm (volume weighted geometric mean diameter, $\bar{D}_{3.3}$ in μm). A small amount, less than 5%, will be in droplets with diameters >10 μm. The droplets are covered by a shell of fat crystals, which prevents the droplets from merging together.[41] Emulsifiers, which are present at the oil/water interface, play an important role in the stability of the structure. The water dispersion introduces an effective microbiological preservation principle: a contaminating microorganism present in a droplet would be restricted in its growth either by space limitations or by exhaustion of the nutrients available in the droplet. This is a feature that margarines have in common with butter, although the latter product may have a coarser emulsion. The importance of fine emulsions for the microbiological stability of margarines is well-known,[25,72] and models have been developed that quantitatively predict the contribution of the emulsion to the microbiological stability of the product.[81–83]

The emulsion characteristics, volume weighted geometric mean diameter ($\bar{D}_{3.3}$ in μm) and geometric standard deviation (e^{σ}),[1,2] of the droplet size distribution can be determined using pulsed-field-gradient nuclear magnetic resonance (NMR).[16,55,61] In the example mentioned above, the $\bar{D}_{3.3}$ is 3.0 μm and the e^{σ} is 2.0. Alternatively, droplet sizes can be determined by microscopical techniques. The contribution of the fine water droplets to the preservation of yellow fat spreads will be discussed in more detail in Section 29.5.

29.3.2 Initial Microflora

The microbiological quality of ingredients should be ensured by purchasing on the basis of microbiological specifications. The quality of a raw material, as obtained from the supplier, can be a Critical Control Point (CCP) in a hazard analysis critical control point (HACCP) plan, if the raw material is at risk of being contaminated with relevant microorganisms and the raw material does not undergo a decontamination step when used in the production of a margarine. In this section, the microbiological issues related to the quality of the raw materials commonly used in margarines will be discussed.

Oil and Oil-Soluble Ingredients

Oil will be virtually sterile because of the high temperatures used during refining. The absence of water in the oil and the transport and processing equipment is a prerequisite to prevent microbiological problems. As water is heavier than oil, water condensate will accumulate at the bottom of tanks or in low, insufficiently flushed parts of process equipment, creating unexpected problems with molds and other microorganisms. This happens only if the oil system is improperly designed. Ingredients added to the oil, eg, flavors, colors, vitamins, and emulsifiers, generally have a very good microbiological quality and will not contribute significantly to the initial microflora of the product. Lecithin, made from soybean oil, can be contaminated with lipolytic microorganisms such as *micrococci* and, occasionally, yeasts.

Dairy and Dairy-Based Ingredients

As the milk from which these ingredients are made is always pasteurized, microbiological criteria can be quite tight, for powders, eg, aerobic plate count of <10,000 cfu g^{-1}, Enterobacteriaceae (as indicator for hygiene) <100 cfu g^{-1}, yeasts and molds <100 cfu g^{-1}, *Bacillus cereus* <1,000 cfu g^{-1}, and absence of infectious pathogens. For liquids, microbiological criteria are an aerobic plate count of <1,000 cfu g^{-1}, *Enterobacteriaceae* (as indicator for hygiene) <10 cfu g^{-1}, thermophilic bacteria <100 cfu g^{-1}, yeasts and molds <100 cfu g^{-1}, *B. cereus* <100 cfu g^{-1}, and absence of infectious pathogens. Buttermilk powder may contain high counts of thermophilic spore-formers, which is important if the stock solution of dairy ingredients or aqueous phase is stored at a temperature allowing growth. *Bacillus cereus* counts should be regularly monitored, in view of the increasing occurrence of *B. cereus* in milk.[27] The number of *B. cereus* may limit the storage time of the stock solution or the aqueous phase as its spores can germinate and grow at a minimum temperature of 4°C. The time and temperature of storage should not allow growth of spore-formers. Because the presence of bacterial spores in the finished product cannot be prevented, the composition and the physical structure of the finished product should limit their outgrowth. Some dairy-based ingredients, notably butter and butter oil, may contain harmless *Microbacterium* spp. and lactic acid bacteria. They both appear as pinpoint colonies on aerobic plate count agar.

Remaining Ingredients

Spices and herbs may be added to a margarine for flavoring purposes; there is a risk that these may be contaminated with pathogens such as *Salmonella* spp. in dry spices and herbs; *L. monocytogenes*, *E. coli* O157, and *Salmonella* spp. in fresh or frozen spices and herbs; and high numbers of spoilage microorganisms including molds and yeasts. Spices and herbs should be decontaminated by the supplier, or prior to processing by the margarine manufacturer, especially if there is no decontamination step after they have been added. Other ingredients such as salt, preservatives, and acidulants are generally free of microbial contamination because of the way they are manufactured. Water must always be of potable quality and drawn from a reliable source.

Packing Materials

The microbiological quality of the packing material is very important for margarines, as the product is cold-filled. The packing material, ie, wrappers, tubs, lids, coverleaves, and bottles, can be contaminated with a variety of microorganisms, the level of which depends on the circumstances under which the packing material is manufactured, stored, and handled. Molds are particularly relevant for margarines, as they are able to grow on the surface of the product if enough moisture and nutrients are available. The contamination level of mold spores on packing material can vary from very clean batches, ie, batches that are almost free from contamination, to batches that are contaminated with up to 5 cfu 100 cm^{-2} of packing material. Clean batches can only be manufactured when specific hygienic measures have been implemented at the source. The inside of the packing material should not be touched by hand because contamination of the packing material will result, with up to 2 to 5 cfu per touch. However, because even specific measures cannot guarantee a contamination-free manufacture of packing materials and the packing materials for margarines are never decontaminated, there is always a risk of mold contamination.

29.3.3 Processing and Its Effects on the Microflora

Overview of Margarine Process

The process is designed to prepare a mixture of (all) ingredients and to convert the mixture into a stable product consisting of an aqueous phase dispersed within a continuous fat phase. The mixture of ingredients is generally prepared via one of two routes, ie, the dual route (Figure 29-2A) or the premix route (Figure 29-2B). In the dual route, the aqueous phase and fat phase are prepared separately and combined at a later stage. The aqueous phase is prepared by adding all water-soluble ingredients, including salt and, if relevant, powdered or liquid dairy material and preservatives to potable water and adjusting the pH, if necessary, to 3.5 to 6.0 using citric or lactic acid. The ingredients may already be dis-

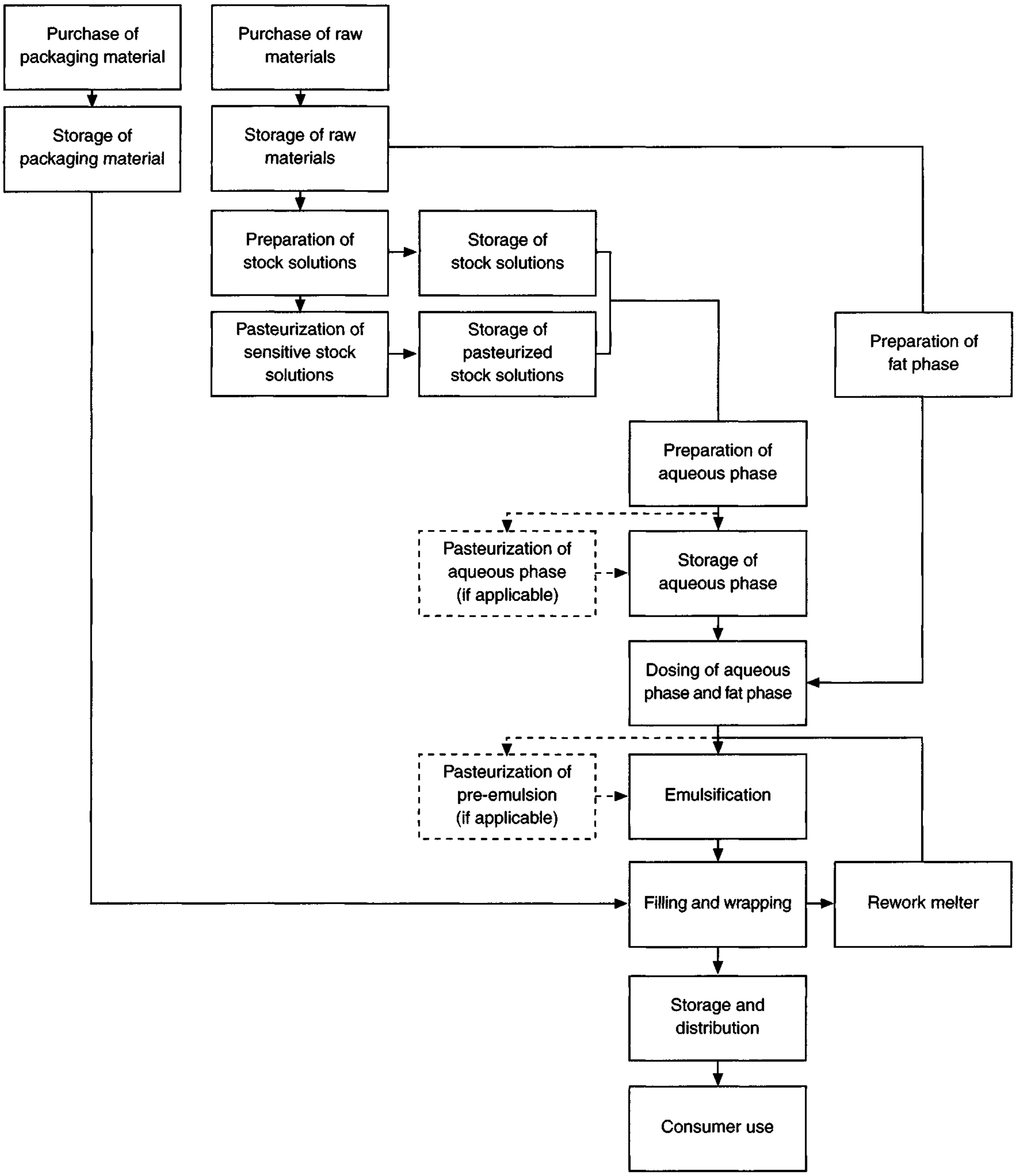
Purchase of packaging material
Purchase of raw materials
Storage of packaging material
Storage of raw materials
Preparation of stock solutions
Storage of stock solutions
Pasteurization of sensitive stock solutions
Storage of pasteurized stock solutions
Preparation of fat phase
Preparation of aqueous phase
Pasteurization of aqueous phase (if applicable)
Storage of aqueous phase
Dosing of aqueous phase and fat phase
Pasteurization of pre-emulsion (if applicable)
Emulsification
Filling and wrapping
Rework melter
Storage and distribution
Consumer use

Figure 29-2A Diagram of the Margarine Production Process in Which the Ingredients Are Mixed via the Dual Route

solved and stored separately as stock solutions. The aqueous phase is stored at 10° to 20°C, depending on the microbiological stability of the composition. The fat phase is prepared by adding oil-soluble emulsifiers, flavors, colors, and, occasionally, preservatives to the fat blend which is stored at 45° to 60°C. To create the pre-emulsion (dual route), the aqueous phase and fat phase are dosed using a proportioning pump (Figure 29-2A). The premix route is slightly different, as stock solutions of ingredients are prepared separately. A premix (or pre-emulsion) is then made by combining the ingredients in a tank using a weighing system (load cells) (Figure 29-2B). From here on the two margarine processes are the same. The pre-emulsion or premix is pumped by a high-pressure pump through a closed system of scraped

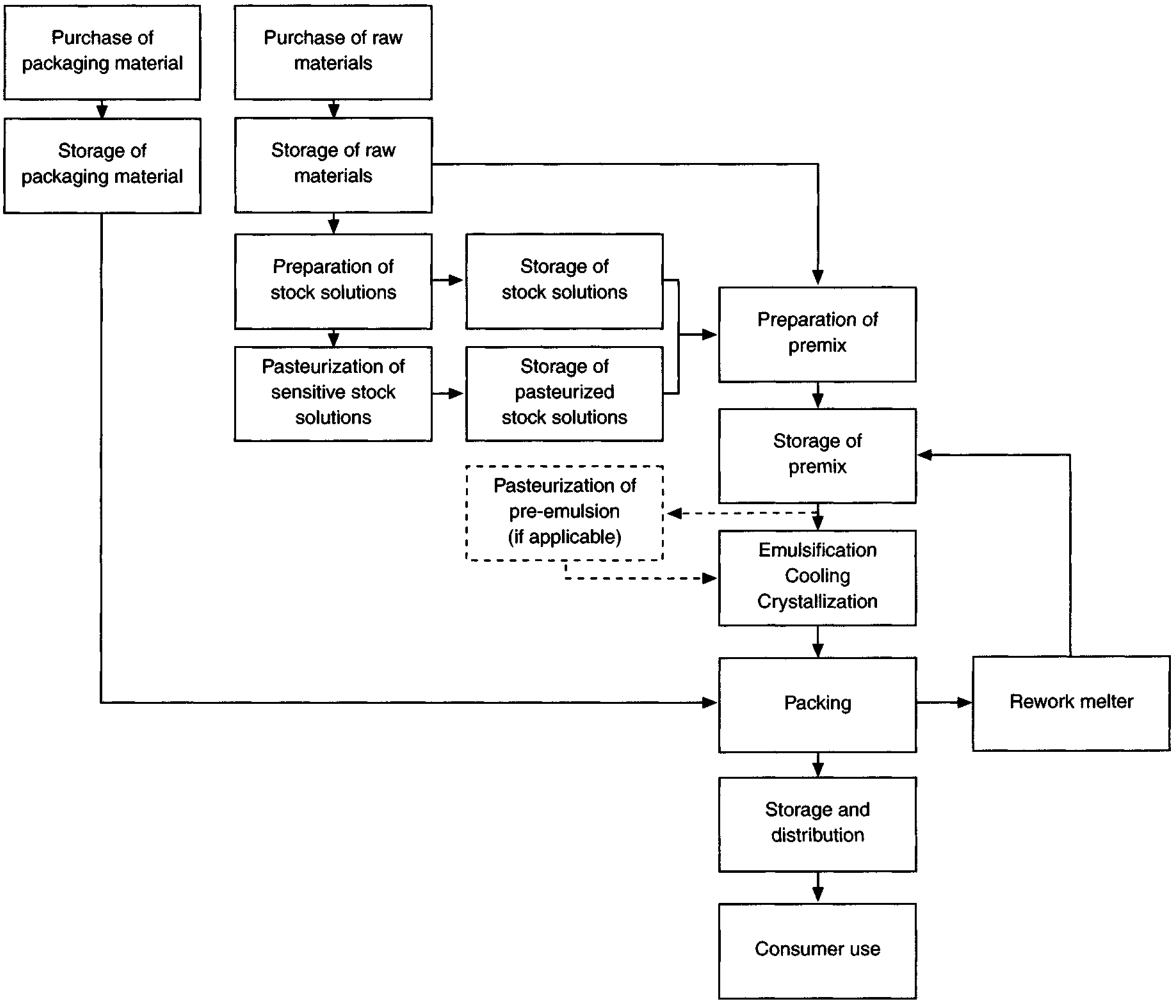
Purchase of packaging material
Purchase of raw materials
Storage of packaging material
Storage of raw materials
Preparation of stock solutions
Storage of stock solutions
Pasteurization of sensitive stock solutions
Storage of pasteurized stock solutions
Preparation of premix
Storage of premix
Pasteurization of pre-emulsion (if applicable)
Emulsification Cooling Crystallization
Packing
Rework melter
Storage and distribution
Consumer use

Figure 29-2B Diagram of the Margarine Production Process in Which the Ingredients Are Mixed via the Premix Route

surface heat exchangers, which cool the product to 10° to 20°C and disperse the aqueous phase to obtain a fine emulsion, after which the fat phase is allowed to crystallize to a certain extent in a crystallizer (pin-stirrer). Hard margarines are packed in wrappers, soft spreadable products in tubs.

Pasteurization is optional. The composition of the aqueous phase will determine whether pasteurization is required. In the dual route, pasteurization may be required at the stage of the stock solutions, the aqueous phase, and/or the pre-emulsion, dependent on the composition of the solutions, the microbial load, the time and temperature of storage, the hygiene of production, and the microbiological stability of the finished product. In the case of margarines, in contrast to low-fat spreads, pasteurization of the aqueous phase is more common than pasteurization of the pre-emulsion. Pasteurization of both the aqueous phase and the pre-emulsion is never done. In the premix route, pasteurization can be applied at the stage of the stock solutions and/or the pre-emulsion. The design of the process should be such that during production pathogenic microorganisms cannot multiply at all and spoilage microorganisms cannot multiply to unacceptable levels.

Any hold-up at the packaging stage will create product that has to be reworked. After secondary packing, the final product is distributed and stored during closed shelf life at ambient or chilled temperature depending on several factors, such as the composition and contamination level (mold spores), factors that are also relevant to the conditions for consumer use. A HACCP study should identify the CCPs and control measures in the process. Preferably, likely CCPs should be identified at the design stage of the product and process (see Chapter 58). Three important aspects of production will be discussed here, ie, pasteurization requirements, hygiene of production, and shelf life.

Pasteurization Requirements

A 6 log reduction of relevant pathogenic and spoilage microorganisms is often defined as the aim of the heat treatment, but other criteria are also used. Whether pasteurization is required depends on the type of ingredients used and their microbial load. For some mixtures, eg, a stock solution of dairy ingredients (a so-called sensitive solution), the minimum pasteurization requirement applied for milk, 15 seconds at 72°C,[32] is sufficient. Generally, more stringent heat treatments are applied, which depend on the target microorganisms and the composition of the mixture. Target organisms can include pathogens such as *L. monocytogenes*, *E. coli* O157, *Salmonella* spp., and *Staphylococcus aureus*, and spoilage microorganisms such as yeasts, vegetative bacteria (eg, Enterobacteriaceae), and molds (mycelium and spores). Bacterial spores will, of course, survive pasteurization. Margarines based on an aqueous phase of only water and salt may not require a pasteurization step. High salt levels (≥8% on aqueous phase, ie, ≥1.4% on product, if the product contains 16% water) may significantly influence the heat resistance of relevant microorganisms.[85] Oils and fats can protect against heat treatment.[8,26,47] For margarines, such a protective effect could present a hazard when relevant microorganisms have to be inactivated at the pre-emulsion (or premix) stage. A protective effect by oils and fats is, however, only found in the absence of water or in the presence of very low levels. Because of the large amount of water present in margarines (15% to 20% on product), it is not expected that there will be a protective effect of oils and fats on the survival of vegetative microorganisms. This has been confirmed by heat-inactivation experiments with relevant pathogenic and spoilage microorganisms, performed in a model oil-in-water emulsion with a similar composition to that of margarines.[85]

Hygiene of Production

The manufacturing of margarines does not always require the same strict hygienic measures. The measures needed depend on the microbiological stability of the intermediate and finished product. Once the multiplication of relevant microorganisms can be predicted at the expected storage and/or distribution temperatures, it is possible to specify the hygienic requirements for the process and the pasteurization requirements to ensure that microbiological limits for such products will not be exceeded during the intended shelf life. This is explained in more detail in Section 29.5. If the process equipment is not sufficiently hygienic and the risk of microbiological problems is thereby too high, corrective measures are necessary. These may include product reformulation.

The preparation of the aqueous phase and the stock solutions are microbiologically vulnerable steps. There may be contamination from the environment and human sources. When temperatures over 40°C are used for extended periods, there is a risk of possible growth of thermophilic microorganisms. Sensitive raw materials, ie, ingredients such as dairy materials and proteins that may harbor spoilage and pathogenic microorganisms, must always be heat treated at some stage in the process. It is common in the margarine process to pasteurize these sensitive ingredients. Occasionally the aqueous phase or the complete emulsion (pre-emulsion or premix) is pasteurized, but the latter approach is more commonly used for the manufacture of low-fat and very low-fat spreads, which are generally more vulnerable to microbial contamination. Of course, it is of utmost importance to prevent recontamination of the pasteurized (intermediate) product as there is generally no further heat treatment in the process.

From the formation of the pre-emulsion onward, the process line will usually be closed, excluding contamination from the factory environment. The equipment can be a source of contamination, eg, of yeasts or spoilage bacteria belonging to the group of Enterobacteriaceae, if improperly designed (eg, dead spaces) or maintained (see Chapter 59). The hygienic state of the equipment is an important factor in determining the time of a production run until the next cleaning cycle.[19] It is often possible, however, to produce more vulnerable products with equipment that does not meet the highest hygienic standards, provided that the line is properly cleaned and disinfected before start-up of production. The time of a production run may then also need to be restricted. A critical step in margarine manufacturing is often the packing of the product. The design of the packing machines may be complex and often they are not cleanable-in-place. Manual cleaning is, however, difficult to control and should be avoided whenever possible. During packing the product is exposed to the environment, thus air quality is important in order to limit contamination of the product with mold spores. If necessary, a laminar air flow cabinet should be used, but this is exceptional in case of margarines. Secondary packing materials may be a source of mold spores, especially if recycled cardboard is used[65] and should preferably not be handled in the packing area. The procedures of storage, transport, and handling of packing materials should reflect the fact that these materials will contain a cold-filled food product. The packing material should not be touched by hand, as this will result in contamination, eg, with mold spores, and thereby increase the risk of mold spoilage.

If the packing machine is mechanically out-of-order or if the product has not been packed correctly, rework of the product may be required. Unpacked product can be reworked through a closed return pipeline and the product can re-enter the main line without a decontamination step, provided that the hygiene is controlled. Otherwise repasteurization is required. Packed product is reworked in a different way; the product is melted and the oil is subsequently purified in the refinery.

Closed Shelf Life and Consumer Use

The temperature of closed shelf life may be ambient or refrigerated, depending on the sensitivity of the product, the contamination level (mold spores), the type of packing material, and/or climatic conditions. From a microbiological point of view, a limitation of the shelf life period is usually not required but the shelf life is generally limited to 3 to 6 months for reasons of chemical stability. Open shelf life conditions are generally similar to closed shelf life conditions. In most cases, it is advisable to store opened packs and tubs in the refrigerator. Contamination of the product during consumer use is likely to occur but growth of contaminating microorganism is expected to be limited in view of the fat-continuous nature of the product, the small size of the water droplets, and, when applied, the low storage temperature. Upon damage of the emulsion, the product can be locally water continuous. Even then there will probably be no problems as the amount of water available may still be limited, there is not necessarily a microbial contamination at that location, and that the contamination, if present, is possibly scraped off again during further usage. In practice, preserved and nonpreserved margarines appear to be very stable products during closed and open shelf life.

29.3.4 Spoilage Associations and Problems Due to Pathogens

Spoilage Associations

Lipolytic yeasts and bacteria such as micrococci and Pseudomonaceae (*Pseudomonas, Flavobacterium*) may grow in coarse or in unstable emulsions. For this reason, in the past many products were tested routinely for lipolytic microorganisms using the Eijkman-plate.[70] Currently, testing for lipolytic microorganisms is less relevant, because major improvements in the hygiene of production and the quality of raw materials have resulted in much better quality products. Lipolytic microorganisms, especially yeasts, occasionally still cause spoilage problems, generally as a result of insufficient hygienic precautions, and can hydrolyze the fat to yield free fatty acids and trigger fatty acid oxidation.

Most spoilage problems in margarines are now caused by molds. In most cases, spoilage is evident as visible mold growth on the product but sometimes it is invisible and the cause of off-flavors or the presence of small water droplets. Mold spoilage of margarines is confined to low-salt varieties, ie, products with a salt level of <8% on aqueous phase (<1.4% on product in the presence of 16% water). Products in which the aqueous phase lacks nutrients because of the absence of a dairy ingredient, or which are preserved with sorbic acid, for example, and have a sufficiently low pH are less likely to be spoiled. Molds in margarine are detected on media such as oxytetracycline glucose yeast extract agar, with the exception of *Cladosporium* spp., which may be difficult to culture.[71,73] If margarines are contaminated with molds, these are mostly spores present at the surface of the product at a level of 1 to 5 spores per tub or package. One spore can cause spoilage of a product. Conditions that favor mold growth include higher temperatures (>10°C) and development of free moisture on the surface of the product. Differences between product temperature and air temperature can induce condensation of water. These conditions are likely to occur during closed and open shelf life and may be due to repeated movement of product in and out the refrigerator. Water can also become available for molds when the emulsion of the product is damaged by a knife, for example. Many mold species can cause spoilage, eg, *Trichoderma* species such as *T. viride*[30] and *T. harzianum*,[85] *Penicillium* species such as *P. roqueforti*[30,85] and *P. expansum*,[30] *Alternaria* species,[30,85] *Paecilomyces variotii*,[85] and *Cladosporium* species such as *C. herbarum*.[30,85] Some molds, eg, *Penicillium* species, can metabolize sorbic acid into *trans* 1,3-pentadiene in a decarboxylation step.[20] Recently, *Paecilomyces variotii* was isolated from a pentadiene-containing margarine and was demonstrated to convert sorbic acid into pentadiene,[66] which leads to an off-flavor described as a "plastic paint" and "kerosene-like"[31] or garlic-like. Because of the strong smell of pentadiene, which is detectable before mold growth is visible, it is very unlikely that the presence of this compound will not be detected by the consumer. Degradation of benzoate to benzaldehyde may also occur, but is less frequently observed.

Occasionally, spoilage may also be caused by Enterobacteriaceae, such as *Enterobacter* spp., generally as a result of a post-pasteurization contamination. These organisms generally do not cause spoilage because growth, if it can occur, is restricted by the continuous fat phase. Enterobacteriaceae, together with yeasts, serve in practice as good indicator microorganisms for the hygiene of a production line.

Problems Due to Pathogens

There are hardly any reported cases of foodborne illness caused by consumption of margarine. A US Food and Drug Administration (FDA) enforcement report mentions a recall due to contamination with staphylococcal enterotoxin from (blended) margarine and butter products.[77] The food poisoning outbreak involved more than 265 cases of illness in two states in southwestern US. In a study of 15 outbreak isolates from patients and food sources, a single, enterotoxin A–producing strain of *Staphylococcus intermedius* was identified as the etiologic agent. *Staphylococcus intermedius* was isolated from a margarine, a vegetable margarine, and different samples of butter blend.[43]

In the UK, the death of an elderly woman from listeriosis was claimed to have been caused by contamination of a margarine with *L. monocytogenes*, and margarine of the same batch was recalled from the market. However, *L. monocytogenes* was not detected in samples of the suspected margarine or of other samples from the same batch, and inspection of the factory did not reveal a risk of contamination of the product from the plant. Furthermore, there was no evidence that the *Listeria* was transmitted by the margarine.[7]

29.4 DAIRY AND NONDAIRY SPREADS

29.4.1 Composition and Structure

Composition

The fat content of water-in-oil dairy spreads and nondairy spreads can vary from 20% to 80%. Below 20% fat, spreads are in general oil-in-water. Common terms used to describe these spreads are "reduced-fat" spread, for products with a fat content of > 41% and < 62%, and "low-fat" or "light" spread, for products with a fat content of ≥10% and ≤41%.[17] Spreads made exclusively from milk fat are named dairy spreads,[17,22] those made from vegetable and animal fats, with a milk fat content of 10% to 80% of the total fat content, are called "blends."[17] Other spreads are called "fat spreads"[17,23] or "nondairy spreads," if they contain ≤3% milk fat of the total fat content.

With a decrease in fat content, the composition of the aqueous phase has become more important for obtaining a spread with a proper physical structure and an acceptable microbiological stability. The first low-fat spreads were based only on water and salt in the aqueous phase, because dairy protein destabilizes the emulsion. These products are microbiologically stable because of the very limited amount of nutrients in the aqueous phase. However, they are often not very tasteful due to the absence of a dairy ingredient (especially the absence of a dairy protein). These products are still on the market, but in addition there is now available a range of spreads containing milk or milk products, based on butter and blended spreads. Thickeners, preservative, acidulants such as citric and lactic acid, vitamins A and D, flavors, and colors may be added. Spreads containing 30% to 50% fat generally require a thickener for reasons of physical stability, with gelatin (1% to 3% of the product) often being the first choice. Spreads with a fat content of 20% to 30% may contain up to 10% carbohydrates, such as starch, alginates, and gums to structure the aqueous phase.

The microbiological stability of the spreads is usually enhanced by adding preservatives; those generally allowed are sorbic acid[18] and benzoic acid or their salts in concentrations of, for example, 0.1% (in spreads with a fat content ≥ 60%) and 0.2% (in spreads with a fat content < 60%). Because undissociated sorbic acid is the major antimicrobial agent, the pH should be sufficiently low, ie, < 5.3 and preferably < 5.0, to ensure the presence of sufficient undissociated acid. A pH of < 4.5 is generally not possible because of precipitation of dairy proteins. The salt level in the aqueous phase is generally lower than in margarine or butter, except for spreads containing 70% to 80% fat. The salt level of the product ranges in general from 0% to 1% and rarely contributes to the microbiological stability of the spread.

The composition of a reduced-fat spread may be 60% fat + emulsifier, 38% water, 1% milk powder, 0.1% citric acid, 0.5% sodium chloride, 0.06% potassium sorbate, and pH 4.9. The composition of a low-fat spread may be 40% fat + emulsifier, 55%

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water, 1% milk or whey powder, 0.1% citric acid, 0.3% sodium chloride, 3% gelatin, 0.12% to 0.16% potassium sorbate, and pH 4.9. The composition of a very low-fat spread may be 20% fat + emulsifier, 71% water, 0.1% citric acid, 0.3% sodium chloride, 8% starch, 0.16% potassium sorbate, and pH 4.9. The composition of a water-continuous very low-fat spread may be 85.9% water, 2% dairy cream (38% fat), 0.1% citric acid, 3% gelatin, 9% starch, 0.16% potassium sorbate, and pH 4.9. To these spreads, vitamins A and D, flavors, and colors are usually added.

Structure

The structure of fat-continuous dairy and nondairy spreads is in principle similar to that of butter and margarine, respectively, except that the aqueous phase may be structured by thickeners and the water dispersion is often coarser. A low-fat spread with dairy protein is more difficult to produce than an 80% fat spread with dairy protein and will generally, but not necessarily, have larger water droplets.[42,52] Continuing improvements in the control of processing conditions make the production of a 40%, protein-containing spread with a reasonable water droplet size distribution, eg, with 50% of the water volume in droplets with diameters < 15 µm and less than 5% in droplets with diameters > 90 µm, possible ($\bar{D}_{3.3} = 15$ µm, $e^{\sigma} = 3.0$). Low-fat spreads with 40% fat and lacking protein are easier to produce and often have a finer water dispersion, eg, with 50% of the water volume in droplets with diameters < 10 µm and less than 5% in droplets with diameters > 45 µm ($\bar{D}_{3.3} = 10$ µm, $e^{\sigma} = 2.5$). Spreads containing 20% fat are now generally fat continuous, but often have a very coarse water dispersion, eg, with 50% of the water volume in droplets with diameters < 50 µm and less than 5% in droplets with diameters > 300 µm ($\bar{D}_{3.3} = 50$ µm, $e^{\sigma} = 3.0$). Besides the water dispersion, the stability of the emulsion plays an important role in the microbiological stability. For example, at the surface of the product where free moisture can become available as a result of coalescence of droplets, microorganisms such as molds can grow. Water-continuous spreads with 3% to 5% fat have a gel-like, spreadable structure, which is often obtained by the use of thickeners such as starch and gelatin.

29.4.2 Initial Microflora

Most ingredients are similar to those used in the manufacturing of butter and margarine (Sections 29.2.2 and 29.3.2), except for thickeners. In general, gelatin is of a very good microbiological quality, because of the way it is manufactured, but may contain thermophilic spore-formers (in low numbers), which can grow rapidly at 50° to 55°C in a stock solution of gelatin in water. The storage time of the gelatin stock solution should not allow outgrowth of thermophilic spore-formers. In addition, the stock solution can be preserved with a low amount of potassium sorbate. Starches are generally also of a good microbiological quality but may contain high numbers of bacterial spore-formers, including *Bacillus*, particularly *B. cereus* in rice starch. Microbiological specifications for starches and gums could be aerobic plate count <10,000 cfu g^{-1}, Enterobacteriaceae <100 cfu g^{-1}, yeasts and molds <500 cfu g^{-1}, *B. cereus* <1,000 cfu g^{-1}, and absence of infectious pathogens. Generally, it is possible to obtain starches with an aerobic plate count <100 cfu g^{-1} or even lower, but this may be dependent on the type of ingredient and the region in the world from which the ingredient is bought. Because the presence of bacterial spores in the finished product cannot be prevented, the composition and the physical structure of the finished product should not allow their outgrowth.

29.4.3 Processing and Its Effects on the Microflora

Processing

As the manufacturing of most dairy and nondairy spreads is not fundamentally different from the manufacturing of butter and margarine, including the water-continuous spreads, the information provided in Sections 29.2.3 and 29.3.3 is also applicable here. There are a few differences. First, dairy and nondairy spreads are in general microbiologically more vulnerable than 80% fat spreads and may require additional measures to produce a safe and wholesome product. Furthermore, in contrast to margarines, the aqueous phase of the pre-emulsion or premix containing < 60% to 70% fat will be present as a continuous phase through part of the process. The aqueous phase will then generally be more vulnerable to growth of microorganisms. Phase inversion, ie, conversion of the water-continuous pre-emulsion into a fat-continuous emulsion, generally occurs at the emulsification step. The aqueous phase of water-continuous spreads is water continuous throughout the whole process. The type of process is similar to the premix route (Figure 29-2B). At the emulsification step, using scraped surface heat exchangers, the premix is converted into a spreadable product.

The microbiological stability of the spread will determine the hygienic requirements of the production process.[44] For vulnerable products, eg, the 40%

fat spread described in Section 29.4.1, in-line pasteurization of the complete emulsion is applied. The pasteurization aims at a 6 log reduction of relevant microorganisms. For elimination of vegetative pathogens, a process of 2 minutes at 70°C or an equivalent heat treatment may be applied. Growth of microorganisms before pasteurization should be prevented because high numbers of microorganisms may not always be inactivated by the pasteurization and, in addition, products resulting from their metabolism might remain in the form of toxins, enzymes, and off-flavor compounds, affecting the stability and safety of the product. It is advisable to decontaminate any potentially suspect raw material before the preparation of the aqueous phase or preemulsion. Interim and finished product should be tested regularly to verify that microbial growth (including that of thermophiles) does not occur during processing. Equipment should be designed to prevent contamination during processing, especially after the last decontamination step, and it should be possible to properly clean and disinfect the process line, which should preferably be done in-line. Proper hygiene during packing is of major importance, because spreads with <70% fat can be very mold sensitive. Some products, such as low-fat and very low-fat spreads and especially water-continuous spreads, may be so vulnerable to mold spoilage that special measures such as a laminar flow cabinet with sterile air may be needed and decontamination of packing materials may be necessary (comparable to aseptic packing of liquid products).

Closed Shelf Life and Consumer Use

Factors determining the storage conditions of spreads are the composition of the aqueous phase, the physical structure, and stability in the case of fat-continuous spreads, the residual contamination with microorganisms after processing, and the expected contamination during consumer use. Dairy and nondairy spreads often require refrigerated storage to guarantee a safe and wholesome product, especially during consumer use. Total shelf life is generally limited to 3 to 6 months, often for reasons other than microbiological. For some products, eg, for unpreserved low-fat spreads containing a dairy ingredient or water-continuous spreads, the duration of consumer use may be limited for microbiological reasons to 2 to 3 weeks, for example. For most spreads the duration of consumer use is, however, not limited. The residual contamination after processing can be predicted, knowing the processing conditions, and thereby largely controlled. Control of contamination resulting from consumer use is more difficult, but is very important. During consumer use the product will become contaminated with a variety of microorganisms originating from the air, bread crumbs, or from other foods. Part of the contamination will, however, be scraped off the surface by subsequent use of the product, thus the contamination is highly dependent on the (frequency of) use of the product.

29.4.4 Spoilage Associations and Problems Due to Pathogens

Spoilage Associations

Most microbiogical problems are due to mold spoilage. The same type of molds are involved as in the spoilage of butter and margarine (Sections 29.2.4 and 29.3.4). Sorbic and benzoic acid greatly reduce, but do not eliminate, the risk of mold growth. As with margarines, some mold strains are able to convert sorbic acid to 1,3-pentadiene but products containing 1,3-pentadiene will probably be rejected by the consumer because of the strong, unpleasant smell. In highly vulnerable spreads mold growth may occur during distribution even if the contamination of fresh product is below the detection limit. This explains why considerable attention must be given to packing hygiene and storage conditions. To date, water-continuous spreads have not shown more spoilage problems than fat-continuous spreads.

Problems Due to Pathogens

No reports of foodborne illness caused by the consumption of dairy and nondairy spreads, including water-continuous spreads, are known. This may be due to the attention given to controlling spoilage bacteria through stricter hygiene, which reduces the likelihood of problems with pathogenic bacteria. Pasteurization of the stock solutions, the aqueous phase, or the complete emulsion is commonly used to control infectious pathogen hazards. In addition, the fat-continuous nature of most spreads, the composition of the aqueous phase, in particular the presence of a preservative, and the refrigerated and sometimes limited storage conditions offer considerable protection against growth of pathogens during shelf life.

29.5 PRESERVATION

29.5.1 Introduction

The preservation of fat- and water-continuous yellow fat spreads relies on a complex, sometimes delicate, balance of a variety of intrinsic and extrinsic factors. Intrinsic factors are the physical structure and the composition of the aqueous phase; extrinsic factors are the temperature and time of storage and the primary packing material. To investigate whether the preservation concept ensures a safe and wholesome shelf life, several studies may be carried out in the laboratory, the factory, and even the household before the product is introduced on the market. Studies in the laboratory may include inoculation studies of the aqueous phase, but also simulation studies of closed and open shelf life. To investigate the risk of mold spoilage during closed shelf life, the surface of the product can be sprayed with a very low level of mold spores, eg, <10 spores 100 cm^{-3}. After incubation at the intended temperature of closed shelf life, the product is inspected visually for mold growth. To investigate the microbiological stability during open shelf life, the surface of the product can be sprayed with vegetative bacteria. After incubation at the relevant temperature, eg, alternating at 7° and 20°C, the product is examined microbiologically to monitor the growth of the microorganisms. Results of laboratory studies can be verified with storage trials of products made in the factory and studies in the household. Microbiological examination of products used at home by the consumer is particularly relevant when a new, sensitive type of product, such as a water-continuous spread, is introduced.

29.5.2 Intrinsic Factors

In fat-continuous spreads, the water is dispersed as small water droplets in the continuous fat phase. Sometimes water channels may be present, eg, in spreads with a very low-fat content (20%), or in butter if not properly made. The compartmentalization of water is important to the microbiological stability.[83] Contaminating microorganisms will be contained in a water droplet, the size of which can be sufficiently small that multiplication of contaminating bacteria or yeasts is physically not possible. This can be important in preventing growth of aerobic spore-formers, which are always present in butter and margarines containing a dairy ingredient, because they survive pasteurization. Growth of microorganisms may also be inhibited or restricted because the supply of nutrients in a droplet may be limited. If abundant growth occurs in a large droplet, this will often be localized. A margarine in which most of the water droplets have a diameter of < 10 μm, with 50% of the total water volume in droplets smaller than about 3 μm (volume weighted geometric mean diameter, $\bar{D}_{3.3}$ = 3.0 μm and $e^{\sigma} = 2.0$, see example in Section 29.3.1) will contain 6.6×10^0 water droplets cm^{-3}. If all droplets are large enough to contain a microorganism, at a contamination level of 10^2 microorganisms cm^{-3}, a maximum of 10^2 droplets per cm^3 would contain a microorganism and multiplication would be confined to those droplets. The sizes of the water droplets can be determined by microscopical methods or by pulsed-field-gradient NMR.[16,55,61]

The physical condition at the surface of the fat-continuous products may differ considerably from that in the bulk of the product. In addition, the microorganisms may be from a different origin, eg, from the packing material or the air to which the product is exposed during packing, or result from consumer use. For these microorganisms the compartmentalization of water is also relevant, but in a different manner. Because of the fat-continuous nature, moisture at the surface will be limited to such an extent that these microorganisms cannot grow unless condensation occurs on the surface. To base the microbiological stability of a water-in-oil emulsion on the water dispersion is of course only valid if the emulsions are physically stable. Physical stability can be ensured by a proper product design, temperature and time during storage and distribution, and proper handling during the whole shelf life. Other intrinsic factors contributing to stability, either alone or in combination, are salt-on-water, pH, preservatives such as sorbic acid and benzoic acid, possibly bacteriocines and diacetyl resulting from fermentation of milk, and limited availability of nutrients; the microbiological stability of water-continuous spreads relies mostly on these factors, in addition to the storage temperature and time. In butter and margarines, the salt concentrations can be high enough to inhibit growth of most microorganisms in the aqueous phase, eg, 2% on product can result in 11% on aqueous phase. More often it is the combined effect of salt and compartmentalization of water that is effective. Salt concentrations of 8% on aqueous phase can retard or inhibit growth of molds at the surface and thereby reduce the chance of mold spoilage, even in those cases where molds can grow in the presence of 8% salt. Yeasts may grow at extreme values of salt concentration and acidity and are difficult to control by the composition of the aqueous phase alone.

The pH is rarely sufficiently low to inhibit by itself the growth of microorganisms. In margarines without a dairy ingredient, the pH can be as low as 3.5. However, even this pH is not sufficient to limit growth of yeasts and molds. Nevertheless, the pH value of the product is critical in many products, particularly when combined with preservatives such as sorbic acid and benzoic acid. Sorbic acid is most frequently used in yellow fat spreads. Undissociated sorbic acid is active against molds, yeasts, and many bacteria, eg, aerobic spore-formers. The pH should be sufficiently low to allow sufficient undissociated acid to be present (the pK_a value of sorbic acid is 4.76). The sensitivity of molds to sorbic acid has been the subject of many investigations.[20,49] The level of preservative required depends on other intrinsic factors, such as the fat percentage, the water dispersion, and aqueous phase composition (including pH), in addition to extrinsic factors such as storage temperature and time. A concentration of 0.033% undissociated sorbic acid on aqueous phase, for example, is considered to be just sufficient to control mold spoilage in a protein-containing, fat-continuous, 40% fat spread (0.12% potassium sorbate on product, pH 4.9) during closed and open shelf life, provided the product is packed in a laminar flow cabinet to prevent contamination with mold spores and the product is stored in a refrigerator ($\leq$ 7°C). Water-continuous spreads may require higher levels of undissociated sorbic acid, ie, $>$ 0.04%, to prevent mold spoilage. Higher levels of undissociated sorbic acid can be reached by increase of the sorbic acid level and/or decrease of the pH. The pH level is, however, generally not much lower than 4.9, because lower values cause precipitation of dairy proteins. Maximum sorbic acid levels are usually subject to regulations. It is important to realize that a large part of the undissociated sorbic acid is dissolved in the fat phase and is therefore not effective as an antimicrobial, as undissociated sorbic acid has an oil/water partition coefficient of 3.3. Although the pH and the concentration of undissociated sorbic acid in yellow fat spreads rarely completely inhibit the growth of bacteria, except the outgrowth of aerobic spores, they are, in combination with other factors such as the physical structure and chilled storage conditions, critical factors in the preservation of many spreads. Growth of many contaminating microorganisms, such as Enterobacteriaceae, which are important contaminants during consumer use, is thereby prevented at the same time. During fermentation of milk with lactic acid-producing cultures, antimicrobial compounds such as bacteriocins and diacetyl may be formed. These compounds influence the growth of certain microorganisms,[38] but their relative contribution to the microbiological stability of yellow fat spreads has not been described. They may play a role in enhancing the microbiological stability, especially in combination with other factors.

The limited availability of nutrients in a compartmentalized system, such as a fat-continuous spread, can contribute considerably to the microbiological stability. Not only can the limited availability determine whether a microorganism can grow, but also whether and for how long it can survive. In the latter case, maintenance energy demands of microorganisms will determine the duration of survival.[67] Predictions of the growth or survival of microorganisms are useful to define hygienic performance criteria for manufacturing of the product, eg, pasteurization conditions and hygiene of equipment, to ensure that microbiological limits will not be exceeded during the intended shelf life. It is possible to describe quantitatively the growth of microorganisms in water-in-oil emulsions. A mechanistic model, which predicts the growth and survival of microorganisms in such emulsions, has been developed.[81] The model is based on the classical Monod equation, which describes the relationship between the concentrations of (growth-limiting) nutrients and the resulting biomass, and on the water dispersion and the distribution of microorganisms over the water droplets. Incorporated into the model are the maintenance energy required by a cell to survive and the effects of growth of microorganisms in a droplet, ie, formation of inhibitory compounds, reduction in space, and decrease in nutrient concentration. Physiological information about each type of relevant microorganism is required to predict the growth factor. The total growth factor of microorganisms in an emulsion is calculated from the growth in the individual droplets. Several assumptions were made in developing the model, among others that microbial growth in a droplet always starts from one cell and that diffusion of water-soluble nutrients through the lipid barriers between the droplets does not occur. The potential of the Verrips model to predict accurately the growth factor of microorganisms in emulsions was confirmed in validation studies: the growth of microorganisms such as *Citrobacter freundii*[82] and *Listeria innocua*[67] in fat-continuous (water-in-oil) emulsions appeared to be comparable to the predictions by the model. The model is not *a priori* applicable to lipolytic microorganisms. Studies with lipolytic micrococci and yeasts in water-in-oil emulsions, however, revealed a strongly limiting effect on growth.[83] Although there is no clear explanation for these observations, two factors are likely to be involved. First, the amount of lipase formed by a few cells in a droplet is possibly too small to provide enough nutrients for growth and, second, hydrolysis of lipids by lipases is inhibited by the coating of the fat with emulsifiers and the physical structure of the fat.[83]

The Verrips model has been proven useful in practice and was incorporated into a computer program for prediction of the stability of margarines, reduced- and low-fat spreads.[44,83] The computer program consists of two parts.[44] The first part predicts whether a selected number of microorganisms, molds excluded, can grow in the aqueous phase. For those microorganisms that are predicted to grow in the aqueous phase, the Verrips model is applied in the second part to predict to what numbers the microorganisms can grow out in the water droplets of the fat-continuous emulsion. Subsequently, the program classifies the products into hazard categories, which are ranked according to the severity of the hazard.[44] The output from the computer program is used as input into the design of the production process and shelf life conditions.

29.5.3 Extrinsic Factors

The most important extrinsic factors are temperature and storage time. The temperature required during closed shelf life to ensure a good microbiological stability will be determined by the microorganisms present in (and on) the product, the effect of intrinsic factors on these microorganisms, and the influence of temperature on the physical stability. There may be a need for (additional) control of the growth of microorganisms by storage at a low temperature (≤7°C), eg, as an extra safety measure or to prevent outgrowth of mold spores that may be present at the surface of the product. In addition, refrigerated storage may often be required to maintain a proper physical structure. During consumer use, many products will require refrigeration as the product will then become contaminated with a variety of microorganisms. Whether refrigeration is critical for the microbiological stability of a product will depend on the physical structure and the composition of the aqueous phase, for example, water-continuous spreads will always require refrigerated storage. In general, the open shelf life of fat-continuous spreads does not need to be restricted in time, because the stability can be ensured sufficiently by other factors. There may however be exceptions. Whether open shelf life needs to be restricted in time will depend on the composition of the product and the volume of the package sold.

The packing material can be of considerable importance. Parchment is often used for wrappers of butter or margarines and in some cases as cover leaves in tub products. If the parchment absorbs water, cellulolytic fungi can grow. Parchment can become wet by condensation of water when the product is transferred from cold storage to a humid and warm environment and back again, but also immediately after production during post-crystallization of the fat phase. The use of parchment impregnated with potassium sorbate is believed to reduce this risk, but this has not been demonstrated. A wrapper that does not contain parchment at the inner side, for example, parchment coated with polyethylene, is probably better to control mold spoilage. Coated parchment has the additional advantage that molds at the outer side of the package cannot come into contact with the product.

29.6 CONCLUDING REMARKS

The manufacturing of yellow fat spreads has undergone major changes during the last decades. More stringent control of hygiene during manufacture had been required for the development of microbiologically stable low-fat products. Improvements in cleaning and disinfection procedures and in the control of the quality of raw materials have been necessary. This has resulted in a range of reduced- and low-fat products with, generally, an excellent microbiological quality and, equally important, an efficient means of producing high-quality butter and margarines. Preservation of many yellow fat spreads relies on a combination of factors such as the compartmentalization of the aqueous phase, stability of the emulsion, composition of the aqueous phase, and low storage temperature. The contribution of water compartmentalization to the preservation decreases when the fat content in the product decreases. Recently, water-continuous products with fat contents as low as 3% have been introduced successfully on the market. These products are developed for spreading on bread as low-calorie alternatives for higher fat spreads. Water-continuous products lack the protective effect of the fat barrier, but this can partly be compensated for by adding more preservative. However, the products are still likely to be more sensitive to microbial growth and, although they are used as if they are a fat-continuous yellow fat spread, it should be realized that their preservation relies on a partly different principle. Up to now, water-continuous spreads have not been associated with microbiological problems, probably because considerable attention is paid to the product design, starting materials, and hygiene of processing.

In the future, special attention should be paid to define those criteria that ensure a safe and wholesome shelf life. A structured approach in evaluating the microbiology of spreads will be helpful to cope with all questions regarding the processing and stability of yellow fat products. Mathematical models describing the behavior of microorganisms in foods can be helpful in evaluating the product and process design, especially at an early stage of development or when defining the HACCP plan. However, the effort to develop and implement the models in daily practice should not be underestimated, in view of the large number of microorganisms, preserving factors, and conditions involved. A thorough understanding of the microbiology of the raw materials and the influence of processing conditions, product structure, and process hygiene on these is required to identify all hazards and risks involved in the production and stability of yellow fat spreads.

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30 Mayonnaise, Dressings, Mustard, Mayonnaise-Based Salads, and Acid Sauces

Martin J.M. Michels and Wil Koning

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30.1 INTRODUCTION

This chapter is about the microbiological safety and stability of oil-in-water emulsions and nonemulsified products with a low pH acidified with acetic acid and other organic acids. Some heat-processed sauces will also be described. The emulsified products are mayonnaise, salad dressings, and oil-containing sauces. Ketchup, vinaigrette, and vinegar-based sauces represent the nonemulsified product types. Additionally, mustard and mayonnaise-based salads are described. The survey concludes with pasteurized acid sauces, for which vinegar is not a characteristic ingredient.

Products containing vinegar have the same major spoilage organisms (acetic acid–tolerant lactobacilli, yeasts, and molds) in common due to a low pH and the presence of acetic acid, although the vinegar is sometimes replaced by lactic or citric acid. In egg-based emulsified products, contamination with infectious pathogens like *Salmonella* and *Listeria monocytogenes* is the main safety hazard. The nonemulsified products have no specific association with pathogens. However, significant microbiological hazards are known for mayonnaise-based salads.

Nearly all these products are intended for repeat use, which sets special demands for their preservation. During consumer use, they may become contaminated with spoilage organisms and even pathogens.

30.2 MAYONNAISE, DRESSINGS, AND OTHER EMULSIFIED SAUCES

30.2.1 Composition

Mayonnaise can be a very well-defined product with specific levels of oil (min. 52%), egg yolk (min. 6%), salt (min. 1%), total acid (min. 0.75%), and pH (max. 4.0) fixed in local regulations, as in Central African Standard S_2.[30] A comparison of some regulations reveals that products with 26% oil (United Kingdom [UK]) and 80% oil (Belgium) can equally be labeled as mayonnaise (Table 30-1). The trend to low-oil products in the 1980s generally has led to reduced oil levels for mayonnaise. This is reflected in current trade practice, which includes clear labeling of fat content and reference on the label to reduced-calorie mayonnaise or similar wording. The types of oil are mainly soybean, rapeseed, and sunflower oil and sometimes cottonseed and olive oil.

The 1989 Codex Alimentarius Regional European Standard for Mayonnaise defined mayonnaise as a condiment sauce obtained by emulsifying edible vegetable oil(s) in an aqueous phase consisting of vinegar, the oil-in-water emulsion being stabilized by the hen's egg yolk.[56] This Codex standard recognized the following optional ingredients: egg white, egg products, sugar, salt, condiments, herbs, spices, fruits and vegetables including fruit juice and vegetable juice, mustard, and dairy products. Acetic acid, citric acid, lactic acid, malic acid, tartaric acid, and the salts thereof were allowed as acidifying agents. Apart from stabilizers, antioxidants, colors, flavors (together with monosodium glutamate as flavor enhancer), benzoic acid and sorbic acid, and the salts thereof were allowed as preservatives. Minimum levels for oil and technically pure egg yolk are given in Table 30-1. A worldwide Codex standard is under discussion and the 1993 draft version specifies a total fat content of not less than 65%.[57]

In 1991, the Association of the Mayonnaise and Condiment Sauce Industry of the European Union (EU) adopted a total fat content of minimum 70% and a minimal egg yolk content of 5%,[35] but these values may be reconsidered.

The United States (US) standards of identity for mayonnaise demand a vegetable oil content of at least 65% together with vinegar and/or lemon juice or lime juice as the predominant acid and egg yolk–containing ingredients.[127] According to Smittle,[116] the pH may range from about 3.6 to 4.0 with acetic acid representing 0.29% to 0.5% of the total product. The aqueous phase may contain 9% to 11% salt and 7% to 10% sugar. The water activity of three brands of regular mayonnaise was measured as 0.93, mainly due to a level of 8% to 9% of salt in the aqueous phase.[32]

Low-fat mayonnaise, reduced-calorie mayonnaise, salad dressing, salad cream, and other emulsified products made with oil, emulsifier, and vinegar typically have a lower fat content than mayonnaise. The salad dressing will have a more fluid consistency to make the product pourable, while most mayonnaise is spoonable. They normally contain egg yolk although some are made with dairy-based emulsifiers. While there is legislation to specify the composition of real mayonnaise in many countries, this is less so for low-oil mayonnaise, dressings, and emulsified sauces.

For salad dressings and French dressing, the US has compositional requirements, but most other countries do not. Salad dressings are defined by the US Food and Drug Administration (FDA) as emulsified semisolid foods prepared from vegetable oils, vinegar, lemon juice and/or lime juice, egg yolk–containing ingredients, and a cooked or partially cooked starchy paste. The finished product contains not less than 30% of edible vegetable oil and has the equivalent of 4% liquid egg yolk.[127] Typically, the pH ranges from 3.2 to 3.9, acetic acid from 0.9% to 1.2% of the total product, and the aqueous phase contains 3% to 4% of salt and 20% to 30% of sugar.[116] Standards are also set for French dressing (not less than 35% oil). In trade practice, these standards are not always followed and labeling a product as French-type dressing gives some freedom for compositional innovation.

Table 30-1 Compositional Requirements for Oil and Egg Yolk in Mayonnaise

Country	*Minimum Oil (%)*	*Minimum Egg Yolk (%)*	*Status*	
Belgium	80	7.5	legal	
Denmark	80	6	semi-official	
France	50	5.6	CoP[a]	
Germany	80	6	CoP	
Great Britain	26	2.7	legal	
Ireland	75	3.5	CoP	
Italy	70	5	CoP	
Netherlands	80	6	legal	
United States	65		legal	
Codex Regional European Standard	78.5	6	advisory	1989
CIMSCEE	70	5	CoP	1991
Codex standard	65		draft	1993

[a] CoP = Code of Practice.

As may be expected, there are few compositional specifications for other emulsified sauces, which are often referred to as dressings or sauces (meat and fish sauces). They generally contain less oil than mayonnaise and salad dressings, but all are oil-in-water emulsions in which the emulsifying agent usually is egg yolk.

Because of the presence of vinegar or other weak acids in most mayonnaise, dressings, and emulsified sauces, their pH is low, which has a significant influence on the microbiological stability. In continental Europe, the pH of mayonnaise typically is between 3.0 and 4.2, with 4.5 as the highest value (the legal maximum in Denmark). A study in the UK, however, refers to pH values up to 4.6–4.8 for commercial full-fat and reduced-calorie mayonnaise made with spirit vinegar and lemon or citric acid.[82] The percentage salt or sugar is not fixed by regulations but is mostly between 1% and 12% of the aqueous phase. The level of acetic acid in the aqueous phase is typically between 0.8% and 3.0%.

Dressings contain less fat than mayonnaise and contain starch, which helps to give the required consistency, resulting in a spoonable or pourable product. Because the aqueous phase contains lower concentrations of acid and sodium chloride than in mayonnaise, dressings are more liable to microbial spoilage. Typically the acetic acid content of dressings is 0.5% to 1.5% of the aqueous phase and the pH 3.0 to 4.2. Levels of salt (1% to 4%) and sugar (1% to 30%) in the aqueous phase contribute little to microbiological stability.

The development of novel oil-free mayonnaise and dressings has led to products that are no longer emulsified. These belong to the vinegar-based sauces (Section 30.3).

30.2.2 Initial Microflora

The microbial load of mayonnaise, dressings, and emulsified sauces comes from the ingredients used to make the product together with any contamination that may originate from the processing and packaging operation.

Microorganisms Present in Oil

The oil used is normally a refined oil that is free of microbial contamination as a result of the refining process, which involves steaming at a temperature well above 100°C. The oil has a very low moisture content of <0.1% and will thus not allow microbial growth. Contamination of the oil during transport or reception at the plant may result in transfer of the contamination to the final product. Due attention to the design, cleaning, loading, and unloading of (dedicated) road tankers will prevent this.

Microorganisms Present in Egg Yolk

Liquid egg in the form of egg yolk, whole egg, or mixtures thereof is used to produce the emulsified products described here. The egg is frequently used in the form of pasteurized liquid egg yolk preserved with salt (8% to 11%) or with salt and potassium sorbate, eg, 92% egg yolk, 7% salt, and 1% sorbate.

Raw egg is liable to be contaminated with various microorganisms of which pathogens are of most concern (Chapter 24). Normally, pasteurized egg is used but raw egg yolk from freshly broken eggs was also used in mayonnaise in past decades. The safety of the product depended on the presence of sufficient acetic acid to inactivate any pathogens before the product was shipped to the consumer (Section 30.2.5).

In view of the risk of contamination of eggs with *Salmonella enteritidis* (Chapters 24 and 45), this practice is now largely abandoned and products are made with pasteurized liquid egg, which is sometimes stated on the label. Pasteurization eliminates pathogens and, in addition, lactobacilli, yeasts, and molds that could act as spoilage organisms. European Community (EC) regulations[5] require that the pasteurized egg is distributed and stored at a temperature of 4°C or less and that *Salmonella* is absent in five samples of 25 g. The Aerobic Plate Count (APC) should not be higher than 100,000 g^{-1} and the maximum number of coliforms 100 g^{-1}. Commercial pasteurized liquid egg products often have counts well below these values. The APC commonly is a few hundred to a few thousand bacteria. The same holds for coliforms, which often are absent in 0.1 g. Occasionally, pasteurized egg products contain *Salmonella* spp. or *Listeria monocytogenes* because of inadequate pasteurization or postprocess contamination.

Microorganisms Present in Vinegar

Vinegar is a further key ingredient of mayonnaise and dressings. The vinegar may be obtained by fermentation from a range of raw materials, eg, alcohol, wine, malt, cider, etc. The acetic acid content of the vinegar typically is about 8% to 11%, but lower levels occur in specialty vinegars, such as those prepared from cider or wine.

Because of the high acetic acid content of vinegar, it is not usually liable to spoilage. It may, however, be contaminated with a very acetic acid–resistant mold, *Moniliella acetoabutans*, which can multiply at 8% to 9% acetic acid.[125] Presence of this mold in vinegar is very rare. Artisanal vinegars such as apple vinegar with low acetic acid levels occasionally may carry spoilage organisms. Vinegar used in mayonnaise, dressings, and emulsified sauces is often pasteurized to eliminate enzyme activity originating from the fermentation process. This ensures the absence of the extremely acetic acid–tolerant molds and of a rare acetic acid–resistant lactobacillus, *Lactobacillus acetotolerans,* which can multiply in the presence of 9% to 11% acetic acid at pH 5.0 and in 4% to 5% acetic acid at pH 3.5.[50]

Microorganisms Present in Mustard

Mustard is a common ingredient in many compositions of mayonnaise, dressings, and emulsified sauces. Because of (typically) 1.8% to 2.5% acetic acid in its formulation, mustard does not contain any infectious pathogens, but contamination with high levels of acetic acid–tolerant lactobacilli is well known. Yeasts are normally not found because of the antimycotic activity of allylisothiocyanate present in many mustard types. This is described in more detail in Section 30.4.

Microorganisms Present in Herbs and Spices

Most emulsified sauces, dressings, and mayonnaise get additional flavor and taste from herbs and spices. These are likely to be contaminated with spoilage organisms and pathogens (Chapter 33). Of particular concern is contamination with *Salmonella,* which is known to occur in many materials, in particular peppercorns.[98] In our experience the incidence rate in raw spices and herbs is about 1%. This makes sampling and testing impractical, so often decontaminated spices and herbs or spice extracts are used.

Microorganisms Present in Dairy Ingredients

The use of dairy ingredients in emulsified products is quite common but most of these are pasteurized by the producer. Fermented products, eg, yogurt and cheese, will have a high load of lactic acid bacteria, and yeasts also may be present. Blue cheeses require special attention due the presence of high levels of acetic acid–tolerant molds and lactobacilli. Soft cheese also may be contaminated with *Listeria monocytogenes.*

Microorganisms Present in Sugar, Salt, and Preservatives

Common ingredients, which normally have a very low microbial load, are sugar, salt, and preservatives like sorbic and benzoic acid or the salts thereof. Sugar produced in small industrial operations may be contaminated with osmotolerant yeasts such as *Zygosaccharomyces rouxii* or *Z. bailii,* which are able to cause spoilage of acetic acid–containing emulsified products.

Microorganisms Present in Water

All food products should be made with potable water, which is normally free of pathogens and acetic acid–tolerant microorganisms.

Microorganisms Present in Lactic Acid, Citric Acid, and Other Acids

Acids other than acetic acid or vinegar may be used in emulsified products. Lactic acid typically consists of a 50% or 80% solution of DL-lactic acid with no significant microbial load. Citric acid commonly is used as acid or as concentrated citric juice (30°Brix) with a low pH of approximately 3.1. Spoilage is prevented by pasteurization of the concentrate (or preservation with sulfite) to eliminate spoilage yeasts and molds. Other weak (eg, malic) or strong (eg, phosphoric) acids that may be used are free of relevant contamination.

Microorganisms Present in Starch

Starch is often used in emulsified sauces, dressings, or mayonnaise to increase the consistency. The starch can be "natural," requiring cooking to 85° to 90°C to set the starch or "instant," in which case the starch does not require any cooking. The microbial load of natural starches and instant starches normally is very low with only a few species of *Bacillus* and *Clostridium* present and no pathogens.

30.2.3 Effect of Processing

The manufacture of mayonnaise can be by a batch or a continuous process. In essence, the process consists of the mixing an oil phase (oil with oil-soluble flavors or preservatives) with an egg phase (egg yolk plus salt) and a water phase (with vinegar and additional ingredients). Sometimes starch is also included to give the product the required consistency.

In a batch process, the liquid (salted) egg yolk is typically mixed in a vessel under stirring with the acid water phase. The oil is added under intensive mixing and the resultant coarse emulsion is usually passed through a colloid mill to achieve the small oil droplets (predominantly 5–10 μm) required for a good consistency. Consistency of spoonable mayonnaise is expressed in Steven's value, measured by recording the resistance in g of a measuring gauge pressed into the product at a fixed speed. Steven's values typically are between 50 and 200 g. For liquid products, the Bostwick value is used, which is the run length in cm of the product, when released in a measuring tray and measured within 30 seconds. Typical values are 5 to 10 cm. The inclusion of (cooked) starch is optional and depends largely on the oil content of the mayonnaise. The freshly made mayonnaise is pumped to a holding vessel, and packaged in glass jars, tubes, buckets, or other containers for consumer/professional use.

In a continuous process, a set of proportioning pumps combines the required amounts of liquid egg, oil, water phase, and optionally a cooked starch phase, which are coarsely emulsified in an emulsifying cylinder (with static and rotating pins) and finally passed through a colloid mill. When particulates are present in the food, the starch phase can be added (through an in-line mixer) directly to the final product buffer vessel. Further details on processing of mayonnaise and dressings were published by Lopez.[84]

In the manufacturing of a mayonnaise, there is normally no technological need to apply a heat process unless elimination of enzymes is required from ingredients like vinegar or spices. These enzymes could break down the starch, if present. The aqueous phase containing ingredients such as herbs or mustard, which could contain pathogenic or spoilage microorganisms, may be pasteurized before mixing. As noted above, egg yolk or other egg products are pasteurized by the supplier before use.

The production of many other emulsified products with a lower oil content, such as low-oil mayonnaise, salad dressing, and thousand island dressings is similar, but most contain (cooked) starch to give the required consistency. Some are simply made by introducing a starch phase into a mayonnaise base. In a dressing with particulates made in a batch process, the particulates can be mixed after the colloid mill with the dressing base. An alternative route to prevent product damage is to omit the colloid mill and to introduce the particulates in the first mixer after preparation of the dressing base.

The overall effect of the mayonnaise, dressing, and emulsified sauce manufacturing process on the initial microbial load of the ingredients used is practically nil, when no heat is applied in the process. All emulsified products described in this section have a continuous water phase and the behavior of the microbial load thereof is not affected by the presence of oil droplets. Whether the organisms present are able to survive or multiply in the product depends on the composition of the aqueous phase, in particular, the acetic acid concentration, pH, salt, and preservatives.

30.2.4 Spoilage Associations and Pathogen Hazards

With respect to spoilage, only those organisms able to survive and grow in these products, with a low pH in the presence of acetic acid or other acids, are considered relevant. Micrococci and bacilli, which have been shown to be present, are not discussed because they cannot grow in this acid environment. Bacilli, nevertheless, are reported to spoil dressings; however, reports cited[79] are over 60 years old. No recent reports are known. The authors have not observed any case of spoilage by *Bacillus* spp. in 20 years; it is their opinion that statements about this are outdated and likely to be incorrect.

Spoilage

Microbial spoilage of mayonnaise, dressings, and other emulsified sauces containing vinegar is mainly caused by a small group of acetic acid–tolerant yeasts and lactobacilli, whereas spoilage by molds is very rare because most molds have a limited tolerance of acetic acid.[117]

Yeasts. Acetic acid–resistant yeasts are a frequent cause of mayonnaise and dressing spoilage. *Zygosaccharomyces bailii* and *Pichia membranaefaciens*, can both multiply in the presence of *ca.* 3% acetic acid,[67,78,123] but the latter yeast requires the presence of oxygen and therefore spoilage only occurs at the surface of these products.[117] These strains are responsible for the majority of the spoilage incidents by yeasts. Other species sometimes implicated are *Zygosaccharomyces rouxii*, *Saccharomyces cerevisiae* and *Candida magnolia*, *Z. bisporus* and *Rhodotorula mucilaginosa*, and *Debaromyces hansenii*. Yeasts may cause spoilage by gas formation or by growing as brownish colonies, which sometimes may appear as small oil-like droplets on the surface of mayonnaise. On occasion only limited growth within the product is observed to approximately 10,000 cells g^{-1} and full development is inhibited, probably by oxygen depletion resulting from oxidation of oils and metabolic action of the yeasts.

Lactobacilli. *Lactobacillus fructivorans* is the main lactobacillus reported to cause spoilage of mayonnaise-like products.[117] In fact, in our experience *L. plantarum* and *L. buchneri* are the lactobacilli most frequently isolated from spoiled products, whereas *L. fructivorans* is more uncommon. *L. plantarum* can multiply to very high numbers without causing evident spoilage, which may result in underreporting. Growth of heterofermentative lactobacilli results in gas formation and a decrease in pH, resulting in visible spoilage.

Molds. Molds rarely present a threat to the stability of mayonnaise and dressings because the majority cannot grow in the presence of 0.5% acetic acid.[67,76] Furthermore, the amount of oxygen present is often limited because of steam capping of jars followed by consumption of oxygen by oil oxidation. Growth of a *Geotrichum* species on the surface of mayonnaise in jars that were not well-closed has been reported.[71] *Moniliella acetoabutans* is a uniquely acetic acid–tolerant mold found in acetic acid preserves; this mold can utilize acetic acid and thus tolerate levels in excess of 5%.[41] Tuynenburg Muys[125] referred to this and other acetic acid–resistant molds such as *Monascus ruber* and *Penicillium glaucum*, which can grow in the presence of 1% and more of acetic acid. *Penicillium roqueforti*, which is present in blue cheese, also belongs to this group. Spoilage by any of these molds is very rare for the reasons stated above.

Pathogens

Salmonella. Mayonnaise has been the cause of many outbreaks of salmonellosis. A major outbreak with 10,000 cases in Denmark in 1955 led to a Danish regulation that the pH of mayonnaise should be below 4.5.[71] Thereafter, there were two additional Danish incidents with *Salmonella*-contaminated mayonnaise from large kitchens. In the first, the pH of the mayonnaise was 5.1 and the count of *Salmonella* was 180,000 g^{-1} at the time of analysis 4 days after the meal.[101] The *Salmonella* was identified as *S. typhimurium*, biochemical type 17. The second outbreak involved 41 cases and 2 fatalities. The mayonnaise for a salad was made with raw egg and 2 days after the event the *Salmonella* count of the mayonnaise was 6 million g^{-1}.[87] Again *S. typhimurium*, type 17 was observed and the pH of the mayonnaise was 6.0.

In 1976 a serious salmonellosis outbreak occurred among passengers on four flights to and from Las Palmas (Spain). There were 6 fatalities, and approximately 500 passengers became ill. *Salmonella typhimurium* phage type 96 was isolated from mayonnaise used on the incriminated flights as well as from a food handler involved in the preparation of the mayonnaise.[43] The pH of the mayonnaise was not mentioned in the World Health Organization (WHO) report on the incident but the menu list referred to mayonnaise, mayonnaise acidified with tomato sauce, and "salsa rosa" (mayonnaise and tomato) suggesting the possibility that the mayonnaise with tomato sauce was not properly acidified and thus allowed growth of *Salmonella.*

These early *Salmonella* incidents were probably caused by *Salmonella* from food handlers or raw egg in combination with an elevated pH of the mayonnaise, which allowed survival and/or growth of *Salmonella*. Such events, however, were rare at that time. It was well known that the inside of duck eggs was likely to be contaminated with *Salmonella* spp. and these eggs were printed with an instruction to cook before use. For sound hen's eggs, *Salmonella* was not considered a significant hazard, although potential contamination with *Salmonella* from the outside of the shell was recognized.

A new wave of *Salmonella* food poisoning was reported in the 1980s due to contamination of hen's eggs with particular phage-types of *Salmonella enteritidis* (see Chapter 24). In Spain, homemade mayonnaise caused a very significant increase in foodborne illness due *to S. enteritidis.* The latter became responsible for 78% of all outbreaks of known etiology.[99] In the UK, homemade mayonnaise made from raw eggs became equally a common cause of infection with *S. enteritidis* (see Chapters 24 and 45).

Similarly, in the US, Argentina, and many other countries, cases of food poisoning due to *S. enteritidis* from homemade or restaurant-made mayonnaise occurred.[49,120] In a serious US outbreak, 404 of the 965 persons at risk (mean age 64.2 year) in a New York City hospital became ill and 9 people died (median age 77.5 years). The source of the incident was hospital-prepared mayonnaise made with raw eggs, and contamination with *Salmonella enteritidis* could be traced back to a farm corporation.[121]

Outbreaks of infection with other *Salmonella* spp. have been associated with the use of raw egg. In the UK, 120 people from a total of 700 reported gastrointestinal illness after eating in the House of Lords.[91] *Salmonella typhimurium* definitive type 49 was isolated from a tartar sauce, made from mayonnaise, remaining from the meal. The mayonnaise was made from fresh eggs, oil, and vinegar. The same *Salmonella* type was isolated from the patients and from hen droppings from the farm that supplied the eggs. A few further incidents caused by *S. typhimurium* are reported in the review by Radford and Board.[104] The use of raw egg for mayonnaise contributed to an outbreak at a wedding reception in 1992, in which 81 guests and 11 catering staff became ill due to *S. enteritidis*. The company and the director were prosecuted and fined.[31]

In 1995, in Uruguay 600 cases of salmonellosis were traced to sandwiches that were made with small batches of mayonnaise. The source of the contamination was *S. enteritidis* from unpasteurized eggs. The company switched to pasteurized eggs thereafter.[11]

Nearly all these incidents were caused by mayonnaise made at home, in institutional kitchens, or in restaurants. It is now well known that *S. enteritidis* can survive for a few days in mayonnaise at 20° or 30°C, when citric acid or a low level of acetic acid is used as acidulant.[77,83] In homemade mayonnaise with 0.1% of acetic acid (0.85% acetic acid in the water phase), *S. enteritidis* survived for 5 to 6 days at 30°C but only for 1 day in commercial mayonnaise with 2.26% acetic acid in the water phase. Refrigerated storage of a homemade mayonnaise at 10°C gave less than a factor three reduction in 9 days, while *Salmonella* was eliminated from the commercial mayonnaise (5 log reduction) in 3 to 6 days.[77] Similar findings were reported by Lock and Board.[82,83] An overview of the inactivation rates of *Salmonella* in mayonnaise is given by the International Commission on Microbiological Specifications for Foods (ICMSF).[72]

The use of pasteurized egg yolk, sufficient vinegar to give typically more than 1% acetic acid in the water phase, and a pH below 4.5 have prevented foodborne illness from industrially produced mayonnaise during the past 40 years. The general public is now more aware of the hazards associated with raw egg, and the use of pasteurized egg may be explicitly declared on the label. Consumer organizations no longer state that the best mayonnaise is made at home from oil, vinegar, and raw egg but they warn against homemade mayonnaise and dressings made with such eggs.[7,68] In some countries, the regulations were changed to protect the consumer, for example, in Spain, where in restaurants, bars, and central kitchens, raw eggs for preparation of mayonnaise and sauces for direct consumption are no longer permitted and where the eggs must be replaced by pasteurized egg products.[90] Additionally, this mayonnaise should be max. 8°C at the point of consumption with a final pH not over 4.2 and be kept for not longer than 24 hours.

Staphylococcus aureus. The inactivation of *Staphylococcus aureus* in mayonnaise has been well studied by Smittle.[116] Because of the low pH of these commercial products and the presence of acetic acid, the organism is unable to grow and is normally of no significance for mayonnaise and dressings. This is comparable to margarine and butter for which only one incident is known.[6] In a Danish incident, however, contamination with 1 million of δ-toxin–producing staphylococci was observed in *Salmonella*-contaminated mayonnaise with an atypical pH of 6.0.[87] Radford and Board[104] refer to two outbreaks caused by *S. aureus*, but the "Stacey's Southend" outbreak was not caused by mayonnaise but by sandwiches with chopped eggs.[4] Details of the other outbreak could not be traced.

Listeria monocytogenes. Contamination of raw egg yolk with *Listeria monocytogenes* has been observed[80] and its behavior in reduced-calorie mayonnaise was studied.[60] In products with pH 3.9 and 0.7% of acetic acid in the aqueous phase *L. monocytogenes* did not grow at 23.9°C and was inactivated by a factor of 10^4 within 3 days. In four commercial mayonnaise products with pH 3.3 to 3.9, *Listeria* inactivation at 26.6°C was directly related to aqueous-phase acetic acid concentrations; at 2.2% acetic acid the pathogen was reduced by a factor of 10^8 in 72 hours and at 0.67% acetic acid in 192 hours.[51]

***Escherichia coli* O157:H7.** Mayonnaise was epidemiologically implicated as the vehicle for transmission in a 1993 outbreak of *E. coli* O157:H7 disease with 62 illnesses in Oregon, US, because of mayonnaise-based ranch dressing, blue cheese dressing, and seafood salad. The victims were mostly adult truckers; only four children were involved.[8,9] The evidence available suggested that the mayonnaise (pH 3.9) was contaminated by tainted meat at the retailer. The bulk mayonnaise as delivered was not suspect.[10] This incident stimulated research on *E. coli* O157:H7 in mayonnaise, but no *E. coli* O157:H7 contamination of pasteurized egg yolk or of the wet processing environment in three processing plants for mayonnaise or other emulsified sauces was discovered by Erickson et al.[53] The primary source for this enterohemorrhagic *E. coli* (EHEC) appears to be cattle. Meat and milk from cattle have been directly linked to many outbreaks, but the Oregon outbreak has shown that recontamination of mayonnaise and dressings with this pathogen may lead to unexpected outbreaks, which thus created interest in the survival of this pathogen in an acid environment. There is another report of a possible outbreak due to mayonnaise contaminated with a nonmotile *E. coli* O101, where 300 people became ill in Eastern Germany in 1988.[29]

E. coli O157:H7 has been shown to be rather resistant to acid pH as suggested by an outbreak associated with consumption of apple cider[21] and by survival in apple cider inoculated with *E. coli* O157:H7,[136] although similar survival is known for *Salmonella*.[65] *E. coli* O157:H7 survived much better than a control strain of *E. coli* at low pH and numbers remained unchanged after 24 hours of incubation in trypticase soy broth at pH 3 and 4.[89] In commercial regular mayonnaise and reduced-calorie mayonnaise dressing, *E. coli* O157:H7 survived 7 days at 23.9°C and survived better than the strains of *Salmonella* and *Listeria monocytogenes* tested.[61]

In studies by the US FDA the following observations were made on the survival of three strains of *E. coli* O157:H7 in the mayonnaise implicated in the Oregon outbreak.[129] When an inoculum of 10^8 cells g^{-1} was mixed with the mayonnaise, pH 3.65, the EHEC strains died off rapidly and survived for max. 72 hours at 25°C, but at 7°C the strains could be recovered up to 35 days. Survival of one EHEC strain at 5°C in four sauces made from this mayonnaise was about 35 days in thousand island dressing (pH 3.76); for seafood sauce (pH 4.38) and blue cheese dressing (pH 4.44), the numbers of surviving EHEC were about 500 times higher at that point in time. In a fourth, mayonnaise-mustard sauce, pH 3.68, the inactivation at 5°C was very rapid as no survival was observed after 5 days; this may be explained by the antimicrobial factors present in mustard (see Section 30.4).

Similar findings were reported for two strains of *E. coli* O157:H7 inoculated into a commercial mayonnaise, pH 3.91, at a level > 10^6 g^{-1} as no survival was observed after 96 hours at 22°C.[105] In a ranch salad dressing at pH 4.51 and 4°C survival was much better (more than 17 days). In this study also a strain of *E. coli* and *Enterobacter aerogenes* were used as references. These strains died more rapidly in the mayonnaise and ranch dressing than the *E. coli* O157:H7 strains, with no survival after 48 hours in mayonnaise nor after 14 days in refrigerated ranch dressing. The mayonnaise had a composition meeting the FDA requirements for pH and acetic acid in products containing raw egg.[128] These regulations were meant to ensure inactivation of *Salmonella* by holding mayonnaise for 72 hours before shipment. Recent studies[53] once more confirmed the rapid inactivation of EHEC in commercial mayonnaise with pH values below 4.0. The conclusion was that intact packages of commercial mayonnaise and mayonnaise dressings pose negligible EHEC contamination and health hazard risks.

Other Pathogens. Foodborne pathogens like *Clostridium botulinum*, *C. perfringens*, and *Bacillus cereus* cannot grow in mayonnaise and dressings at a pH below 4.6 and thus should be of no significance. Nevertheless, reference is made to an outbreak in the UK caused by *B. cereus* (and *Staphylococcus aureus*).[104] The pH of the product involved may have been above 4.6, thus allowing growth of these pathogens. *Campylobacter jejuni* has been found in egg yolk but the organism is not heat resistant, is unable to grow at ambient temperature, and is likely to be inactivated rapidly in the presence of acetic acid. One incident was reported in the Netherlands of campylobacteriosis due to a homemade mayonnaise,[3] but details of the incident were not included.

30.2.5 Preservation

The production of mayonnaise, dressings, and other emulsified sauces should be carried out in such a way that contamination with pathogens is prevented and that no spoilage of the final product occurs. A good product design therefore addresses the safety as well the stability of these products. During the product and process design phase and the implementation of the new compositions in an actual manufacturing process, the principles of the Hazard Analysis Critical Control Points (HACCP) system (Chapter 58) should be followed.

Product Safety

Because of the use of egg, the pathogens associated with emulsified products are in particular *Salmonella*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Verotoxigenic *E. coli* might also be considered a pathogen of concern. Two approaches are needed to ensure product safety: (1) to adopt measures to ensure that viable infectious pathogens are not present in the product at the point of consumption and (2) to formulate the product in such a way that pathogens cannot multiply in the product, if contamination occurs.

Absence of Infectious Pathogens. For the industrial manufacture of mayonnaise, dressings, and other emulsified sauces the absence of infectious pathogens from the products can best be ensured by the use of pasteurized egg and pasteurization of all other components that may present a microbiological hazard such as some dairy ingredients or herbs and spices. Pasteurization of egg is used in nearly all industrial production and sometimes a second pasteurization of the egg is applied directly before processing to ensure product safety, as a very low incidence of *Salmonella* and *Listeria monocytogenes* may occur in commercially available pasteurized liquid egg. Such a low level of contamination cannot be detected by endpoint testing using any reasonable number of samples (Chapter 60).

Pasteurization of potentially contaminated ingredients can be done as a separate processing step for herbs and spices, for example, or this can be integrated in the manufacturing process itself. Such in-plant pasteurization is, however, not always applied for the ingredients for which a pathogen hazard may exist. An alternative is to buy all hazardous ingredients from approved suppliers, who control the pathogen hazard by a validated HACCP-based system.

A further approach may be to accept some potential contamination of ingredients and to rely on the pH and composition of the product to eliminate the pathogen hazard. This concept is best illustrated by past practice in the US. Making use of the intrinsic microbial safety of properly formulated mayonnaise and salad dressings is an approved practice in the US, where according to the federal regulations raw eggs or raw egg yolk–containing ingredients may be used, provided that the pH of the dressing is not above 4.1, and the acidity of the aqueous phase expressed as acetic acid is not less than 1.4%.[128] The final product must be held for 72 hours before it is shipped to consumers. This practice has been shown to eliminate *Salmonella* from mayonnaise, when contaminated raw eggs were used, but the use of raw eggs was discontinued commercially in the early 1970s.[60] The US regulation is based on studies on survival of *Salmonella* and *Staphylococcus aureus* in mayonnaise and salad dressings.[131] In these studies, rapid inactivation of *Salmonella* at room temperature or at 37°C within 1 to 6 hours was shown for salad dressing (pH 3.2–3.3; *ca.* 1% acetic acid) and within 1 to 18 hours for mayonnaise (pH 3.8–4.0; *ca.* 0.5% acetic acid). *Staphylococci* survived under these conditions for up to 4 days.

These findings are still relevant to (re)contamination of pasteurized egg or mayonnaise with *Salmonella*. For reduced-calorie mayonnaise, the use of 1.4% acetic acid in the aqueous phase of the mayonnaise would be organoleptically unacceptable, but the elimination of *Salmonella* occurs at 0.7% acetic acid.[60] In typical American reduced-calorie mayonnaise, *Salmonella* and *L. monocytogenes* were inactivated by 4 log in 3 days in products with a pH below 4.1 and 0.7% acetic acid in the aqueous phase. An investigation in the UK with 24 varieties of commercial mayonnaise with pH values of 2.6 to 4.8 and various types of acids showed rapid inactivation of *Salmonella enteritidis* PT 4, when this organism was inoculated into the products at a level of 2.5×10^4 g^{-1}.[82] In eight examples no *Salmonella* could be recovered after 48 hours from products stored at 20°C and *Salmonella* was undetectable after 20 minutes in a fat-free mayonnaise with pH 2.6. At 4°C, *Salmonella* survival generally was longer. In the paper the *Salmonella* inactivation is expressed as $\log_{10}$ decimal reduction times (DRTs). The $\log_{10}$ DRTs (in minutes) for products with a pH of *ca.* 3.7–4.7 were *ca.* 2–3.5 at 4°C and *ca.* 2.5–3.5 at 20°C.

A safety evaluation similar to that of Wethington and Fabian[131] was done by Tuynenburg Muys.[125] He studied survival of Enterobacteriaceae in mayonnaise and dressings after inoculation with *ca.* 5×10^4 cells ml^{-1} of *Enterobacter aerogenes* and *E. coli*. When after 3 days at 20°C the number of Enterobacteriaceae was reduced by a factor 1,000 (so-called 3D concept) the products were declared safe with respect to Enterobacteriaceae. Later, the safety analysis with respect to Enterobacteriaceae was developed into a formula in which pH, undissociated acetic acid, sugar, and salt were incorporated to predict inactivation of *Salmonella* on the basis of the aqueous-phase composition of the product.[36] Because of the widespread use of pasteurized egg and the incidence of new pathogens like *Listeria monocytogenes* and EHEC, this formula has lost most of its significance as a tool to predict safety of new formulations.

The more resistant *E. coli* O157:H7 survived for 7 days in commercial mayonnaise.[61] Inoculation of 6.5 × 10^3 *E. coli* O157:H7 in the commercial mayonnaise, pH 3.6–3.8 and 0.37% titratable acid, implicated in the 1993 Oregon outbreak (Section 30.4) showed survival for 8 to 21 days at 20°C and 34 to 55 days at 5°C.[137] Presence of *E. coli* O157:H7 in raw materials for mayonnaise and other emulsified sauces is, however, not likely, so this is not a major point to be considered during the development of a new product.[53]

Prevention of Growth of Pathogens. To prevent growth in emulsified sauces of pathogens, which might get into the product during use by professionals (catering) and consumers, it is recommended that a composition is chosen that does not permit growth of any pathogen.

Salmonella spp. can multiply down to a pH of 3.8,[72] so pH alone will be inadequate in most emulsified products to stop multiplication of this pathogen, but the content of acetic acid will also contribute to preservation. This will stop multiplication at a much higher pH. For three strains of *Salmonella* the minimum pH for growth in an optimal medium with acetic acid was 5.4, but it is not possible from the data given to deduce the level of acetic acid present.[33] In reduced-calorie mayonnaise of pH 4.3

with 32.7% oil, 0.1% potassium sorbate, and only 0.1% total acetic acid (in the aqueous phase) no growth of *Salmonella* occurred and 10^6 cells of eight *Salmonella* strains were eliminated within 1 week at 23.9°C.[60] The survival of *S. enteritidis* in homemade mayonnaise acidified with vinegar and lemon juice was studied by Perales and García.[100] In a mayonnaise of pH 5.0 containing 0.65% acetic acid in the aqueous phase (calculated from information in the paper) and thus 0.23% undissociated acetic acid, no growth of *S. enteritidis* occurred within 120 hours at 24° or 35°C. In the mayonnaise acidified with vinegar to pH 4.5, *Salmonella* survived well at 24°C, but at 35°C the numbers declined by a factor of 10^6 in 120 hours. In mayonnaise acidified to pH 5.0 or 4.5 with lemon juice, good growth of *Salmonella* was seen at both 24° and 35°C.

The possible growth of *S. aureus* and toxin production in homemade mayonnaise was recently studied in a rather unusual experimental design, where the mayonnaise was diluted with 62.5% sugar syrup (2:1). The latter must have reduced the acetic acid concentration of the vinegar in the mayonnaise by about four-fold.[62,63] Toxin was found only in mayonnaise acidified with vinegar with a pH $\geq$ 5.0. At pH 4.9 and less no growth took place and no toxin was formed. The concentration of acetic acid in the water phase of the mayonnaise in these trials could not be estimated.

For *Listeria monocytogenes*, the minimum pH for growth at 30°C of 16 strains, isolated from patients and outbreaks, in a laboratory medium acidified with HCl was 4.4–4.6.[59] It would be expected that the limiting pH for growth would be significantly higher when acetic or lactic acid was used as acidulant. In TSB, acidified with acetic acid, growth was observed at pH 5.0 for two out of four *L. monocytogenes* strains, while at pH 4.5 a level of 0.044 M (0.26%) undissociated acetic acid inhibited growth in TSB and cells were reduced by a factor 1,000 within a week at 30°C.[38] In reduced-calorie mayonnaise at pH 4.3, 0.1% of acetic acid in the aqueous phase and 0.1% potassium sorbate, *L. monocytogenes* did not grow at 23.9°C but was reduced by a factor 10^6 within 1 week.[60] In a laboratory medium at pH 4.4 to 4.6 with 0.2% acetic acid (corresponding with *ca.* 0.15% undissociated acetic acid) no growth of two strains of *L. monocytogenes* was observed at temperatures from 7° to 35°C.[2] Cells survived at this level of acid, but were inactivated with 0.3% acetic acid. For lactic acid and citric acid, the findings were very similar with 0.2% (no growth) and 0.3% (inactivation), but addition of 0.1% acetic acid was clearly more inhibitory than citric acid, with lactic acid at an intermediate position.

For *E. coli* O157:H7, the minimum pH for growth is probably above pH 4.0.[136] In All Purpose Tween broth acidified with acetic acid to pH 5.0 (but no concentration given) no growth was observed in 70 days,[44] but in brain heart infusion broth at pH 5.0 acidified with lactic acid visible growth of *E. coli* O157:H7 was evident in 2 to 3 days at 25°C. In a detailed study,[37] the growth and survival of *E. coli* O157:H7 at 4°, 10°, 25°, and 37°C in Tryptic Soy Broth with yeast extract (TSBYE) acidified with acetic, citric, lactic, malic, and mandelic acid to pH 4.0, 4.5, 5.0, 5.5, and 7.0 was determined. These studies showed growth at 37°C at pH 5.2 but not at pH 4.5, when pH was adjusted with acetic acid. When the pH was adjusted with citric or lactic acid, these pH values became pH 4.5 (growth) and 4.0 (no growth). At 10°C and pH 5.5, only pH adjustment with acetic acid prevented growth of EHEC as all other acids tested gave good growth within 21 days. The no growth–growth threshold level for acetic acid in the TSBYE medium at pH 5.2 was given as 7 mM undissociated acetic acid, which corresponds with 0.042% undissociated acetic acid. Studies in microtiter plates at 30°C with a range of inocula (10 to 10^5 cells per well) with a mixture of five *E. coli* O157:H7 strains at pH values from pH 3.8 to 5.5 and undissociated acetic acid levels from 0.0% to 0.05% gave complete inhibition of EHEC for 28 days at all inoculation levels in Trypticase Soy Broth at pH 5.1 and 0.04% undissociated acetic acid. At pH 4.6, the growth was prevented by 0.035% undissociated acid, corresponding with 0.06% total acetic acid.[76] With the same levels of undissociated lactic acid, good growth of *E. coli* O157:H7 was seen for all conditions tested.

Based on the above data and the information that EHEC is considered to be one of the more acetic acid–tolerant pathogens, the following product safety rule with respect to pathogen growth has been formulated by the authors of this chapter: "No growth of pathogens will be possible, when the emulsified products have a maximum equilibrium pH of 4.5 and at least 0.2% undissociated acetic acid in the aqueous phase." This corresponds, depending on the pH, to roughly 0.2% at pH 3.0 to 0.3% at pH 4.5 total acetic acid in the aqueous phase, which is well below the level used in most commercial products. When all the acetic acid is replaced by lactic acid or citric acid, the rule is no longer applicable as lactic acid and citric acid are much less inhibitory to *E. coli* O157:H7, *Salmonella,* and *L. monocyto-*

genes.[1,33,37,76] Additional information on the antimicrobial effects of weak organic acids is given in Chapter 8 and in the review of Smittle.[116]

Product Stability

The presence of more than 0.2% undissociated acetic acid in the aqueous phase of an emulsified sauce will control pathogen growth, but this level is not enough to prevent the growth of acetic acid–tolerant spoilage organisms. Some yeasts and lactobacilli can grow in the presence of up to 3% undissociated acetic acid. It is, however, not essential that products do not permit microbial growth. Emulsified sauces should reach the consumer with a low microbial load, and products should not spoil after opening when handled according to the instructions on the pack. This objective can be met in various ways as will be explained later.

The low pH of 3.0 to 4.5 and the preservative effect of undissociated acetic acid (mostly added as vinegar), lactic acid, or other weak acids is most important for stability. In full-fat mayonnaise, salt and to a much lesser extent sugar can reduce the water activity significantly, which may help to inhibit spoilage organisms. Because of a limited water content, the salt concentration in the aqueous phase can be as high as 12% resulting in a water activity (a_w) of about 0.92. The typical compositions of mayonnaise reported by Smittle[116] have 9% to 12% salt and 7% to 10% sugar in the aqueous phase, which will significantly affect growth of lactobacilli and to a lesser extent yeasts. For dressings, an a_w of 0.91 is reported to be inhibitory for *L. fructivorans* and a value of 0.89 for *Zygosaccharomyces bailii.*[88] The combination of pH and water activity for the yeast and lactobacillus gave inhibition of both organisms by the combination of pH (3.3 to 4.1) and a_w (0.85 to 0.89/0.93). For *L. plantarum,* the inhibiting a_w for growth in the presence of salt was 0.943 to 0.960, whereas *Saccharomyces rouxii* still could grow at an a_w <0.868.[106]

In addition to pH, salt, and acetic acid, preservatives like sorbic acid can be used where legally allowed, but a major proportion of the acid will dissolve in the oil phase. According to our calculations for dressings with pH 3.8–4.5 and fat levels of 20% to 80% on average only 30% to 60% of the sorbic acid will be present in the active undissociated form in the aqueous phase to protect against yeast (and lactobacilli). When benzoic acid is used, an even larger proportion of the preservative dissolves in the fat phase, which makes it not very effective. The permitted levels of sorbic acid and benzoic acid within the European Community[13] are summarized in Table 30-2.

Table 30-2 Preservatives Permitted in the EU in Emulsified and Nonemulsified Sauces, Salads, and Mustard

	Maximum Level (mg kg^{-1} or mg l^{-1} as appropriate)[1]			
Foodstuff	*Sorbic Acid*	*Benzoic Acid*	*Sorbic + Benzoic Acid*[2]	*Sulfite*[3]
Emulsified sauces with a fat content of 60% or more	1000	500	1000	
Emulsified sauces with a fat content less than 60%	2000	1000	2000	
Nonemulsified sauces		1000		
Prepared salads		1500		
Mustard, excluding Dijon mustard			1000	250
Dijon mustard			1000	500

[1] The maximum levels of the preservatives sorbic and benzoic acid are expressed as the free acid.

[2] This means that sorbic acid and benzoic acid may be used singly or in combination.

[3] Sulfite is expressed as SO_2.

The preservatives may be added as sorbic acid (E 200), potassium sorbate (E 202), calcium sorbate (E 203), benzoic acid (E 210), sodium benzoate (E 211), potassium benzoate (E 212), or calcium benzoate (E 213).

Sulfite may be added as sulfur dioxide (E 220), sodium sulfite (E 221), sodium hydrogen sulfite (E 222), sodium metabisulfite (E 223), potassium metabisulfite (E 224), calcium sulfite (E 226), calcium hydrogen sulfite (E 227), or potassium hydrogen sulfite (E 228).

Source: Adapted with permission from Anonymous, Common Position (EC) No. 26/98 adopted by the Council on 23 March 1998 with a view to adopting Directive 98/... /EC of the European Parliament and Council of ... amending Directive 95/2/EC on food additives other than colours and sweeteners, *Official Journal of the European Communities,* C161, pp. 29–32, © 1998; and Anon, Annex III of European Parliament and Council Directive 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners, *Official Journal of the European Communities,* No. L 61, pp. 16–22, © 1995, European Communities.

Stability Evaluation. The stability of newly developed emulsified sauces is often not easily established and challenge studies with typical acetic acid–adapted strains of lactobacilli, yeasts, and molds were introduced by Tuynenburg Muys.[125] He demonstrated that only a limited number of spoilage organisms were suited to grow in the typical ecological niche presented by these products. An inoculation technique, which was part of the so-called Microbiological Composition Analysis, was used to test the stability and safety of products. For stability testing various strains were used of *Lactobacillus buchneri*, of yeasts (*Pichia membranaefaciens* and *Zygosaccharomyces bailii*), and of molds (*Monascus ruber* and *Penicillium glaucum*).

With a set of typical acetic acid–resistant organisms (*Moniliella acetoabutans, Saccharomyces acidifaciens [= Zygosaccharomyces bailii], Pichia membranaefaciens,* and *Lactobacillus buchneri*) data were collected by Tuynenburg Muys[125] to construct a spoilage-prediction chart for dressings. This chart was an extension of the preservation-prediction chart of Bell and Etchells[19] for the spoilage of sweet cucumbers by *Zygosaccharomyces globiformis (= Torulaspora delbrueckii)* with acetic acid and sugar as variables. The chart of Tuynenburg Muys[125] used the percentage of undissociated acetic acid in the water phase and the amount of salt and sugar (expressed as grammolecules). The prediction chart was later transformed into a formula to estimate the stability of new formulations. In these predictions *Moniliella acetoabutans*, which is extremely resistant to acetic acid, was not taken into consideration, otherwise no emulsified product could be made stable as this mold can grow at 8% to 9% undissociated acetic acid. In practice this mold is extremely rare and can be dealt with by pasteurization of natural vinegars used.

The stability formula is based on *S. acidifaciens (= Z. bailii)*, the next most acetic acid–tolerant organism in the chart. The formula with the growth-limiting concentration for acetic acid, salt, and sugar for pH values between 3.0 and 4.5 is as follows[35]:

$$\Sigma\ 15.75 \times \text{undissociated acetic acid (\%)} + 3.08 \times \text{salt (\%)} + \text{hexose (\%)} + 0.5 \times \text{disaccharide (\%)} = 63$$

In this formula all percentages are w/w, based on the weight of the solutes in the aqueous solution, ie, water plus acetic acid plus salt plus sugars. For acetic acid containing sauces with a stability factor, Σ, above 63, stability was predicted, whereas products with a value below 63 might be vulnerable for spoilage.

An additional development was the creation of a safety formula, which was derived from a number of challenge tests, as described before in the safety evaluation:

$$15.75 \times \text{undissociated acetic acid (\%)} + 3.08 \times \text{salt (\%)} + \text{hexose (\%)} + 0.5 \times \text{disaccharide (\%)} + 40\ (4.0 - \text{pH}) = \Sigma s$$

Products with values for Σs above 63 would be intrinsically safe with respect to pathogenic Enterobacteriaceae, indicating that these would die quickly (factor 1,000 reduction in 3 days at 20°C). Because of the use of pasteurized egg, this formula, which is comparable to the rules developed in the US for mayonnaise with raw egg, lost its significance and safety is better ensured by raw material selection or pasteurization.

The derivation of the stability formula was one of the first attempts to model microbial growth in a food product. In retrospect, the formula is rather fail safe. The formula would require, for example, presence of 4% undissociated acetic acid or 1% undissociated acetic acid and 15% salt in the aqueous phase to stop microbial spoilage, when no significant levels of other inhibitors would be present.

The formula has been used for some time to predict stability of new formulations and still may be applied for some products. However, the requirement for less acidic products with lower concentrations of vinegar and salt has resulted in the need for additional methods of preservation, including the addition of sorbic acid, pasteurization, and refrigeration.[35] Improved control of hygiene during production also contributes to the stability of the products.

Product Design for Emulsified Sauces. Three stability classes may be distinguished:

1. **Stable products.** These products are stable at ambient temperatures, not sensitive to spoilage, whether open or closed, because of an aqueous phase composition that inhibits growth of all relevant spoilage organisms, in particular acetic acid–tolerant lactobacilli, yeasts, and molds. Their closed shelf life will be 6 months to 1 year, limited for organoleptic reasons only. After opening, the sauces are not sensitive to spoilage and they can be kept at ambient temperatures or chilled for as long as their quality remains acceptable.
2. **Unstable products.** These products allow (slow to rapid) growth of lactobacilli and/or yeasts, but good process design and hygienic practices can eliminate or prevent contamination during

manufacturing, allowing long ambient shelf life in the closed jar (6 months to 1 year). Recontamination during consumer use restricts the shelf life of the product after opening to a few weeks (ambient) or months (chilled).

3. **Fresh, chilled products.** Because of minimal built-in preservation, these products have only a short chilled shelf life; contamination should be kept at a low level and chilled distribution is needed to give the required shelf life of a few weeks.

Water Phase Composition. To judge the microbiological stability of emulsified sauces, it is important to know the aqueous phase composition of the product. This may be measured analytically, but often the composition of the product can be calculated when the composition of all ingredients is known (% water, fat, acetic acid, salt, preservatives, etc).

For the antimicrobial effects of acetic acid and other weak acids used as preservatives, the undissociated form of the acid is important. This level can be calculated once the total amount present, the pH, and the dissociation constant, pKa, are known. It is important, however, that a large part of the undissociated acid may dissolve in the oil phase and be lost for preservation. For acetic acid this is a minor part as the partition coefficient oil/water is about 0.03 to 0.07 (average value 0.05). For sorbic acid this coefficient is 3.3, while for benzoic acid most of the undissociated acid goes to the oil phase due to a oil/water partition coefficient of 6 to 13 (average 10).[26]

In the Netherlands, Kneteman developed the formula below (not published) to calculate the true aqueous phase composition. Similar formulae were published later[85,126,133]:

$$C = \frac{C_w \times (\%\text{water} + \%\text{acid})}{100} + \frac{C_w \times \alpha \times D \times \%\text{oil}}{0.9 \times 100} \qquad (1)$$

In which:

C = total concentration of acid in the product
C_w = concentration acid w/w in aqueous phase
α = undissociated part of the acid
D = partition coefficient oil/water of the undissociated acid
0.9 = specific weight of the fat phase

α can be derived from:

$$\alpha = \frac{1}{10^{(\text{pH} - \text{pK})} + 1} \qquad (2)$$

Because the undissociated acid is the inhibitory factor, equations (1) and (2) can be combined to calculate the percentage of undissociated acetic-, sorbic-, or benzoic acid in the aqueous phase as:

undissociated acid, % w/w =

$$\alpha \times \frac{100 \times \%\text{acid}}{\%\text{water} + \%\text{acid} + \dfrac{\alpha \times D \times \%\text{oil}}{0.9}} \qquad (3)$$

As lactic acid (HLa) does not dissolve in oil the percentage of the undissociated form of this acid is calculated by using the formula below:

HLa undissociated, % w/w =

$$\alpha \times \frac{100 \times \%\text{HLa}}{\%\text{water} + \%\text{HLa}} \qquad (4)$$

For the above acids the partition coefficients oil/water and pKa values are given below based on[26]:

	D	pKa
Acetic acid	0.05	4.76
Benzoic acid	10	4.19
Lactic acid	0.0	3.86
Sorbic acid	3.3	4.76

Once the aqueous phase composition of an emulsified sauce is known, the stability can be estimated. This is best done for each of the main types of spoilage organisms (as they differ in their susceptibility against the common preservative factors).

Molds rarely will present a problem as those normally present are unable to grow because of the presence of elevated levels of undissociated acetic acid (>1.5%) or the presence of a preservative like sorbic acid. Yeasts are more difficult to inhibit as they require over 3% undissociated acetic acid in the aqueous phase or an inhibitive combination of a lower level of acetic acid and a preservative such as sorbic acid. They are not affected by a low pH.

Lactobacilli are affected by low pH, by a reduced water activity (high salt or sugar levels), and by undissociated acetic acid. Sorbic acid is less effective against lactobacilli than against yeasts, but it may contribute to stability.

The preservation potential of the weak acids found in emulsified sauces is quite variable. Citric acid generally has no specific antimicrobial activity against the typical spoilage flora of emulsified sauces and its main function is to reduce pH. Lactic acid (undissociated) has the same preservative power as acetic acid (undissociated) against lactobacilli, but its activity against yeasts is much less.[133] The effect on molds is marginal. Most studies on antimicrobial activity of weak acids were, however, done with bacterial pathogens (see Chapter 8).[116] In these studies, acetic acid proved to be more inhibitive than lactic acid, with minimal inhibition by citric acid. Similar results were recently obtained for *L. monocytogenes*[38] and *E. coli* O157:H7.[37]

Limited general guidance can be given about the best preservation for the large variety of emulsified sauces on the market as these range from perfectly stable products to rather vulnerable fresh mayonnaise.

For microbiologically stable products, a process design based on a cold process may be adequate, but an infectious pathogen hazard should be prevented by application of pasteurization for critical ingredients like liquid egg (yolk), as explained earlier.

For microbiologically unstable mayonnaise and dressings, the process design should control contamination hazards originating from ingredients and the environment. Products that are not very sensitive to spoilage may be made with clean ingredients (free from relevant spoilage organisms) under Good Manufacturing Practice conditions based on the General Principles of Food Hygiene of Codex Alimentarius.[55]

More sensitive products may require decontamination of all suspect ingredients, for example, by inclusion in a water phase, which is pasteurized. The process line itself should be free of relevant microbial contaminants at the start of the process. The use of hygienically designed process equipment and packaging machines is the key to the production of such vulnerable products. For those products, a process line that can be cleaned-in-place (CIP) is preferred. This allows the best control over cleaning and disinfection. Proper manual cleaning may still be required for traditional colloid mills, fillers, and other equipment that are difficult to clean by CIP alone because of crevices in the equipment construction, for example.

For chilled products, hygienic processing and clean (decontaminated) ingredients are important, together with proper refrigeration of the product. Equipment traditionally used for the manufacture of mayonnaise and dressings has not been easy to clean, but recently hygienic mixers, colloid mills, valves, pumps, and filling machines that can be "cleaned in place" have become available and their use is recommended. The glass jars, tubes, or other containers used for packaging mayonnaise and dressings are normally free of acetic acid–resistant spoilage organisms, and thus there is no need for decontamination or for extremely clean packaging materials.

For fresh, chilled emulsified sauces, the aqueous phase composition should be known to be able to deduce what the effect would be of chilled distribution and storage on the shelf life. When the chilled chain is of good quality it is likely that yeasts become the limiting factor for shelf life because lactobacilli growth is significantly inhibited by temperatures below 10°C.

Consumer use is a final point to consider. Many mayonnaise and dressings are only spoiled by acetic acid–resistant organisms, which are not widespread in the consumer's home. Consequently, these products will not easily spoil during consumer use. When, however, mild compositions with low acetic acid levels are put on the market, the possibility of spoilage during use should be considered. Such spoilage can be controlled largely (but not completely) by limiting the shelf life of opened product at ambient temperatures or by recommending chilled storage after opening. Label instructions are thus important for these products.

30.3 KETCHUP, VINAIGRETTE, AND OTHER NONEMULSIFIED SAUCES

30.3.1 Composition

The main nonemulsified sauces are ketchup and vinaigrette. Ketchup is made with tomatoes, sugar, salt, vinegar, water, and spices like onion, garlic, cinnamon, and cayenne, but no oil.[94] Normally at least 6% of the dry matter should come from fresh tomato or tomato paste. Sugar or corn syrup form the major part (20% to 30%) of the total solids. The high sugar and high dry matter content in ketchup results in a reduction of water activity to levels of 0.93–0.95. The amount of vinegar added typically results in 0.8% to 1.0% of acetic acid in the product. The spices may be replaced by oleoresins or extracts. Often starch is added to give the product the required consistency, however, this is not permitted in all countries. In those countries, the product with starch is often labeled as a tomato sauce. The pH of ketchup is normally between 3.5 and 4.0. Ketchup may be preserved with benzoic acid or sorbic acid to give sufficient open or closed shelf life. The closed shelf life is about a year at ambient temperature, while the open shelf life can be weeks to months at chilled temperature, depending on product composition and presence of preservatives.

Vinaigrettes are nonemulsified dressings of vinegar and oil for use on green salads. The oil is not present as a true emulsion, but gums are added to produce a temporary or semi-permanent or permanent colloid, which behaves like an emulsion. In two-phase vinaigrette the oil and water phases are separated, and consequently, the product requires shaking before use. Spices and condiments are added to give flavor to the product.

The most commonly used oils are soybean, sunflower, rapeseed, olive oil, corn, cotton, or grape oil. The vinegar may be derived from alcohol, wine, cider, malt, or many other sources. The gums commonly used are xanthan, locust bean gum, carrageenan, gum arabic, guar gum, and others. The herbs and spices used could be tarragon, chives, pepper, and chili, while onion may be added as a powder or as particulates. Mustard is often present. The pH of vinaigrettes is typically 3.0–3.5. They often have a high level of vinegar and thus acetic acid, which will contribute significantly to microbiological stability, but preservatives are sometimes also required.

Ketchup and vinaigrette are two representatives of a large spectrum of nonemulsified sauces. A further example is salsa, which like ketchup is tomato-based but the concentration of vinegar is lower and may go down to 0.1% to 0.2% acetic acid. Fat-free imitation mayonnaise is a further example of this group of products.

30.3.2 Initial Microflora

The initial flora of ketchup, vinaigrette, and other nonemulsified sauces with vinegar depends largely on the raw materials used and originates from herbs, spices, tomato, and onions. Tomato paste is preserved by heat and is free of vegetative spoilage organisms (and pathogens). The paste can be provided in cans, drums, or aseptically packed in bags, which after opening are sensitive to recontamination, and lactobacilli may develop when held after opening. The microflora of vinegar, herbs and spices, starch, and mustard is discussed in Section 30.2.2. Vegetables such as onions can carry elevated levels of lactobacilli, but sometimes also yeasts or molds.

30.3.3 Effect of Processing

Normally, ketchup is made by mixing all ingredients, which are then cooked to 85° to 90°C to set the starch, usually followed by hot filling into bottles. This eliminates contamination with spoilage organisms from raw materials or the manufacturing process itself. Because of the hot fill, contamination during the filling operation will be eliminated. In case of heat-sensitive, plastic, up-side-down bottles a cold-fill process has been applied. In the latter operation, it is particularly important to prevent recontamination of the cooked and cooled ketchup with spoilage organisms from the processing and filling equipment. Hygienic design and proper cleaning and sanitation are essential.

Most vinaigrettes are processed without pasteurization of the aqueous phase because the gums used for stabilization of the oil droplets do not require a cooking step. Raw material selection, pasteurization of components, or choice of compositions that are high in acetic acid are required to ensure the safety and stability of these products.

30.3.4 Spoilage Associations and Pathogen Hazards

Ketchup is often sensitive to spoilage by lactobacilli; Dakin and Radwell[40] refer to *L. brevis*, *L. mannitopeum*, and *L. plantarum* as spoilage organisms identified in studies of tomato ketchup. The lactobacilli are controlled by cooking and hot filling at temperatures above 70°C. In products filled at lower temperatures, lactobacilli are the predominant spoilage organisms. Lactobacilli can be present in spoiled products in the form of large white spots or as small, hard, white dots, which may easily be mistaken for undissolved ingredients or clumps of starch. The lactobacilli can grow with or without gas formation. In the former case, swelling of plastic containers will be seen. Explosion of glass bottles with ketchup is a hazard, particularly when yeasts would spoil the ketchup. Reports on spoilage by yeasts, however, are few.[39,119] The acetic acid–tolerant yeasts that may cause spoilage of ketchup are the same as those that spoil mayonnaise because these products have the same associative flora.[124] Restaurant-made taco and enchilada sauces with a pH of 4.5 to 4.7 spoiled with lactobacilli, yeasts, and molds because of high initial contamination from spices and honey and recontamination of the cooked sauce by unhygienic practices.[48] The use of ethylene oxide–decontaminated spices, pasteurized honey, proper cooking, and prompt chilling gave a chilled shelf life of 2 weeks.

Spoilage of vinaigrettes is less common because of built-in preservation factors, such as a low pH and relative high acetic acid concentration, which give good open and closed shelf life.

Because of the absence of liquid egg and dairy ingredients in ketchup and vinaigrette, there is no direct association of pathogens with these sauces. Herbs and spices may introduce pathogens such as *Salmonella*, but these will be killed by the cooking process of the ketchup or rapidly inactivated by the typically high level of acetic acid in vinaigrettes.

There have been no reports of foodborne illness resulting from these sauces.

30.3.5 Preservation

For safety, a minimum of 0.2% undissociated acetic acid and a pH below 4.6 will be adequate to prevent growth of pathogens (see Section 30.2.5). Such pathogens may originate from raw materials or may get into the sauces during consumer use. At acetic acid levels below 0.2%, growth of pathogens should be considered, although pathogen growth on occasion may be inhibited without vinegar as shown for *Salmonella*, *S. aureus*, *E. coli*, and *B. cereus*. These pathogens were inactivated within 10 days when inoculated into a tomato-based Mexican hot sauce, pH 4.1, made from water, tomato puree, canned hot chili peppers, and salt.[54] The stability of cooked sauces such as ketchup is based on a low pH, reduced water activity because of sugar and acetic acid in combination with either a hot fill or a cold, hygienic fill without recontamination. Cold-prepared vinaigrettes often have a rather low pH of 3.0 to 3.5, which inhibits many lactobacilli, and an elevated level of acetic acid of 1.0% to 2.0%, which provides a fair degree of built-in stability.

Preservatives such as sorbic acid or benzoic acid can be used to improve the closed or open shelf life in combination with refrigerated storage after opening. The above-mentioned tomato-based Mexican sauce spoiled because of yeasts. However, addition of 0.05% of either sorbic acid or benzoic acid prevented yeast growth, when incubated at 25°C for 8 hours and then at 5°C for 16 hours for a period of 10 days.[54] In this investigation no reference was made to lactobacilli but it is possible that these equally were present as the "total counts" of spoiled sauces were over 10^7 ml^{-1}. Whether the added preservatives were adequate to inhibit lactobacilli as well was not investigated.

In the EU 1,000 mg kg^{-1} of sorbic or benzoic acid is permitted for nonemulsified sauces (Table 30-2), but in our experience such levels are not adequate to give complete inhibition of lactobacilli in ketchup and additionally acetic acid or other factors may be required for effective preservation. For ketchup, benzoic acid is often more effective than sorbic acid because it is almost equally active against lactobacilli and yeasts, while sorbic acid is less so against the lactobacilli.[133] In vinaigrettes with a low pH of 3.0 to 3.5, lactobacilli may be inhibited by pH and sorbic acid may be best suited to give the required stability with respect to yeasts.

30.4 MUSTARD

30.4.1 Composition

For centuries mustard has been one of the most important spices used in many foods. The word mustard is derived from the Latin word "mustum" or "must," the juice of grape or other fruit. In the ancient Roman-occupied Britain must was mixed with powdered mustard seeds and used as a paste called "mustum ardens" (hot must).[114]

Dry mustard seed does not have a distinct aroma. The typical sharp pungent taste of mustard develops as soon as the seed is soaked in water. Mustard can be produced from different varieties of seeds. Nowadays, these seeds are mainly cultivated in Canada. Depending on the pungency of the product required the seed variety is chosen. If produced from brown (*Brassica juncea*) or black (*Brassica nigra*) mustard seed, a very hot mustard like "Moutarde Dijon," will be the result. If produced from white or yellow mustard seed (*Sinapis alba*) a much milder product, "Moutarde Douce," is obtained. Combination of the various types of seed results in intermediate products.

The concentration of seed in the different paste varieties varies from 17% to 30%. Legislation for Dijon mustard specifies that it is should be produced from *Brassica juncea* only and that the product should not contain more than 2% of the husks of the grains; furthermore, the dry matter content should be at least 28%. For other varieties, the husks are equally milled in. Besides the grains, the product contains water, salt, vinegar, spices, and, depending on the local taste, sugar or sometimes honey. Typical compositional values for mustard are as follows: acetic acid between 1.8% to 2.5%, salt content between 2.5% to 6.5%, fat content 4% to 8%, a total dry matter 18% to 28%, and a pH of 3.8–4.3.

30.4.2 Initial Microflora

The initial microflora of mustard is mainly a reflection of the microbiological contamination of the mustard seeds, water, vinegar, salt, sugar, and the spice mixtures used and the hygienic status of the equipment. Mustard seeds can be highly contaminated. In a production line of a mustard mill contamination levels of aerobic and anaerobic spore-formers were 10^2 to 6.10^4 and 10^3 to 5.10^4, respectively, and lactobacilli were at levels of 4.10^3–4.10^5.[75] The contamination level of the brown and black seeds generally is higher than of the white seeds. In a survey to determine the microbiological quality of spices and herbs,[98]

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coliforms and *B. cereus* could be found and, at a rather high level, yeasts and molds were present on mustard seed. The detection of *Salmonella* in mustard seed is very difficult and high dilution ratios (1:100) for mustard pre-enrichment broth are needed.[98] For the contribution of the contamination level from the other ingredients see Section 30.2.2.

30.4.3 Effect of Processing

Depending on the type of mustard being manufactured, the seeds used for making the product are either *Brassica juncea* or *Sinapis alba* but mostly a mixture of both varieties is used. German mustard is predominantly made of white seeds, whereas Dijon mustard is manufactured using *Brassica juncea* only. (Since the second world war, the use of *Brassica nigra* is abandoned and replaced by *Brassica juncea.*)

In the manufacture of German mustard, after removing the impurities from the mustard seeds, the whole seeds are added to a mixture of water, vinegar, spices, and salt and left to swell under constant stirring. Then the mixture is coarsely ground, and subsequently, the mixture is left to ferment as a result of enzymes released by the grinding process. The seeds can also be crushed dry and then mixed with water, vinegar, and salt and left for fermentation. The time for fermenting varies from 30 minutes to several hours. The mixture is then passed through corundum stone mills and ground to the desired fineness. The ground mustard is left to mature for a certain period of time, several days to a few weeks, and afterwards packed.

For the manufacture of Dijon mustard, the husks are sieved off because this product should not contain more than 2% husks. The temperature during the whole process should not be too high, especially when *Brassica juncea* is used, because the component allylisothiocyanate (AIT), which is released as soon as the seeds come into contact with water, is volatile. If the temperature gets too high the level of AIT might be too low. Therefore, in the more modern production facilities the corundum stones are cooled to avoid excessive heating. The AIT is responsible for the typical sharp pungent taste of Dijon mustard. The AIT is not present in the seed itself but it is formed as a result of enzymatic hydrolysis of the glucoside sinigrin and the enzyme myrosinase in a watery environment. Both the sinigrin and the enzyme myrosinase are present in the brown or black mustard seed.[81,114]

Mustard produced from *Sinapis alba* does not contain allylisothiocyanate. The pungency of this type of product is caused by the glucoside sinalbin, present in the white mustard seed, which is converted into p-hydroxybenzylisothiocyanate in a watery environment by the enzyme myrosinase. The p-hydroxybenzylisothiocyanate is less pungent than allylisothiocyanate, not volatile, and very unstable.

English mustard is traditionally produced by a different process. Mustard flour, which has been previously produced from dehusked seed by a dry roller milling process, is blended with other precooked ingredients. The product is acidified by citric acid and contains about 9% salt.

It will be clear that the product will not undergo any heat treatment, otherwise the enzymatic reaction will stop. Consequently, the contamination of the product itself will not be eliminated during processing. This means that the contamination originating from the ingredients and the plant will determine the microbial load of the product.

30.4.4 Spoilage Associations and Pathogen Hazards

Only acetic acid–resistant microorganisms are likely to develop in the product and cause spoilage. Although spore-formers (aerobic and anaerobic) will be present, the spores will not be able to germinate and grow because of the low pH in combination with the level of acetic acid. Other bacteria might be present but these will be inhibited and most likely decrease in number in a product with this composition, especially during the maturation period.

The most likely spoilage organisms for mustard are lactobacilli, yeasts, and molds. Some lactobacilli, especially *L. plantarum* and *L. buchneri,* are rather acetic acid resistant. Species that are heterofermentative thus can cause blowing of the product and > 10^6 g^{-1} lactobacilli have been reported in blown mustard.[75] Yeasts in general are inhibited because of the presence of the allylisothiocyanate. Nevertheless *Zygosaccharomyces bailii* was reported as the cause of spoilage of mustard produced by a Czech food company.[28] However, no information was given about the type of mustard, its pH, or acidity. Spoilage of mustard by molds is very unlikely because of the rather high level of acetic acid.

It is generally stated that AIT present in mustard is inhibitive toward growth of yeasts. Specific data are limited. When 5% Dijon mustard was added to malt broth, the number of yeasts was reduced by a factor 1,000 within 7 days incubation at 25°C. This level of mustard corresponds to 0.007% AIT in the broth. To get the same effect with mustard manufactured from *Sinapis alba,* 10% was needed.[76] This study was carried out in malt broth at pH 3.8 and 4.3. All combinations were adjusted to the same level of acetic acid (0.45%) and salt (1.27%). In another experiment, 0.01% AIT in Potato Dextrose broth at pH 4.3 without acetic acid did not inhibit yeasts, but in combination with 1% acetic acid 0.004%, AIT did inhibit the growth during an incubation period of 4 weeks at 25°C.

The addition of 0.3% to 1.5% w/w mustard to mayonnaise resulted in increased death of *S. enteritidis.*[104] Kanemaru studied the effect of AIT and mustard on the behavior of a number of bacteria. It was demonstrated that 0.8% mustard was bacteriostatic for *E. coli* and *S. aureus* and 0.2% was bactericidal for *Pseudomonas aeruginosa*. Growth was inhibited for 24 hours for *S. aureus, E. coli, Proteus vulgaris, Pseudomonas fragi,* and *Pseudomonas aeruginosa* at levels of 0.138%, 0.104%, 0.064%, 0.043%, and 0.089%, respectively. The corresponding levels for AIT were (in mg kg^{-1}) 14.5, 12.3, 6.5, 3.6, and 7.2.[74]

Isshiki studied the inhibitory action of AIT vapor against bacteria, yeasts, and molds. He showed that a reduction of 4 to 5 log could be achieved for Gram-positive bacteria and *S. enteritidis* at a concentration of 100 ng ml^{-1} and for Gram-negative bacteria at a level of about 50 ng ml^{-1}. Yeasts were affected at a level of 16 to 37 ng ml^{-1} and molds at 22 to 62 ng ml^{-1}.[73]

Because of the combination of low pH and the presence of a high level of acetic acid, growth of pathogenic bacteria will not be possible, and if pathogens are present, they will probably die off during the maturation of the product. This is supported by the absence of food poisoning incidents related to mustard.

30.4.5 Preservation

In the EC legislation on food additives[13] sulfur dioxide is allowed in mustard at a maximum of 250 mg kg^{-1} for all other types than Dijon mustard, which may contain 500 mg kg^{-1}. Sorbic and benzoic acid are permitted in mustard to a maximum of 1,000 mg kg^{-1} for the sum of both ingredients (Table 30-2).

The typical composition of mustard with its low pH and presence of > 1% undissociated acetic acid in the aqueous phase, together with the AIT present in most mustard types, is inhibitory for all pathogenic and most spoilage organisms. Lactobacilli can cause spoilage in mustard,[75] but most of the products will also be inhibitory to this organism because of the combined effect of the acetic acid and salt in the aqueous phase. To inhibit the growth of lactobacilli in those mustard types that will allow growth, benzoic acid (Table 30-2) can be added. Benzoic acid also controls the growth of yeasts. Benzoic acid is more effective than sorbic acid against lactobacilli. In historical times mustard was used as a preservative in the wine industry and for fruit juices.[46] This is still the case in Turkey for fruit juices.

30.5 MAYONNAISE-BASED SALADS

30.5.1 Composition

Mayonnaise-based salads or dressed salads are mixtures of a variety of foods with a mayonnaise base. The components may consist of chicken, meat, egg, fish, shellfish, potato, vegetables, herbs, pasta, fruits, or nuts. Apart from the mayonnaise and the main components mentioned, starch, sugar, spices, organic acids, preservatives, and flavor/colors may be present. A specific definition does not exist. Because of the vinegar of the mayonnaise base or the additional acids used, the dressed salads typically have a pH between 4.0 and 5.5. The acetic acid level in the aqueous phase is much lower than that of mayonnaise itself, and is often between 0.2% to 0.5%. Most mayonnaise-based salads are kept refrigerated to extend their shelf life. The chilled shelf life of industrial products typically is between 2 and 8 weeks.

30.5.2 Initial Microflora

The initial microflora of mayonnaise-based salads is a made up of the microbial load of the raw materials used and much depends on the microbiological status of the materials selected for the manufacture of dressed salads. The mayonnaise base typically has a low microbial count with no or a very limited contamination with yeasts and lactobacilli. Otherwise, acetic acid–tolerant spoilage organisms would be introduced with this key raw material with resultant loss of shelf life.

Considering the range of possible raw materials used, quite a variable microbial contamination may be present as shown in previous chapters and in Section 30.2.2. This was documented in a study, where *Bacilli* possibly from vegetables, in particular *B. pumilis, B. subtilis,* and *B. licheniformis* were detected in commercial salads, including chicken, ham, and potato salads, coleslaw, and pimento cheese spread, together with a range of lactic acid bacteria consisting of various species of *Lactobacillus* (*L. plantarum, L. leichmanii,* and *L. casei*), *Leuconostoc* (*L. mesentoroides, L. paramesentoroides*), and *Streptococcus.*[122]

The objective should be to use raw materials that do not introduce pathogens or significant numbers of spoilage organisms into the product. Some materials will have already a low microbial load because of pasteurization or sterilization (meat, egg, poultry, vegetables). Chicken meat, for example, can be pasteurized and delivered chilled to the processing plant, while other pasteurized materials may be put in an acid brine or salted (vegetables, cucumbers) to give them ambient stability.

For a range of natural, raw materials it is difficult to obtain these with a low count (hand-peeled cooked shrimps, vegetables, herbs and spices) and measures should be taken to reduce their counts. This can be done before arrival at the plant (steam decontamination of herbs and spices or use of spice extracts) or by treating materials at the plant. When it is not possible to eliminate spoilage organisms (for example, yeasts and lactobacilli in raw herring), it must be ensured that infective pathogens are absent by selection from safe sources. The composition and shelf life of the salads should be designed to control the expected initial contamination.

30.5.3 Effect of Processing

Mayonnaise-based salads are made by mixing (ambient or chilled) ingredients with a special thick mayonnaise or dressing. Some ingredients such as chicken or meat are cooked, diced, and chilled or bought already chilled and diced. Raw vegetables such as cabbage are well trimmed, washed, and sometimes marinated in special brines with acetic acid and preservatives to reduce their microbial load. Potatoes, carrots, and onions may be diced, blanched, or steamed and chilled. Pasta is cooked, drained, and chilled. Canned products such as fish, fruits, and tomato are chilled, taken out of the can, drained, and roughly minced. The processing of a mayonnaise-based salad essentially consists of a mixing of all ingredients with the mayonnaise base. Normally this does not include a heating step. The processing will not reduce the microbial counts and may even lead to increased counts when times and temperatures permit microbial growth. Particularly when processing is not done hygienically, this may lead to the build-up in the plant of an acid-adapted spoilage flora resulting in contamination of product and loss of shelf life.

The complete salad is packed in tubs or in larger trays for sale by the retailer (counter salads), which are closed with a sealed foil or a snap-on lid. When the salad is made from chilled components in a chilled environment, the product can be case packed, palletized, and put into chilled storage directly. Otherwise, additional chilling of the salad before palletization may be required.

30.5.4 Spoilage Associations and Pathogen Hazards

The concentration of acetic acid (from vinegar) and pH are the major contributors to the antimicrobial properties of mayonnaise-based salads.[69] These factors should be selected so that, in combination with refrigerated storage, the growth of pathogens is prevented. Acid-tolerant organisms, however, may grow and limit the shelf life of mayonnaise-based salads. In some cases pathogens have been shown to cause outbreaks of illness due to consumption of these products.

Spoilage

Yeasts, lactic acid bacteria, and molds are the most frequent cause of spoilage of stored salads because of the relatively low pH. Characteristic spoilage yeasts in coleslaws are *Saccharomyces exiguus* and *Saccharomyces dairensis*.[25] Many other yeasts such as *S. cerevisiae, Pichia membranaefaciens, Zygosaccharomyces bailii, Z. rouxii, Sporobolomyces odorus, Trichosporon beigelii, Torulaspora delbrueckii, Candida sake, Candida lambica, Candida vini,* and *Yarrowinia lipolytica* also have been shown to grow in salads.[17,22,24]

The most frequent lactic acid bacteria that multiply in dressed salads are *L. plantarum, L. buchneri,* and *L. brevis*. Less common types are *L. leichmannii, L. delbrueckii, L. casei, L. fructivorans, L. confusus, Leuconostoc mesenteroides,* and *Pediococcus damnosus*.[17,52] All of these organisms come primarily from vegetables and pickles or the processing environment. The combined use of sorbic and benzoic acids can inhibit yeasts, but these preservatives have much less effect on lactobacilli.[69] Multiplication of lactobacilli in mayonnaise-based salads may lead to high counts, which are not always accompanied by spoilage. Gas-producing lactobacilli or rope-forming strains produce more evident signs of spoilage. Molds produce visible spoilage, whereas yeasts produce off-flavor, gas formation, or colonies visible as surface growth.

A significant aspect for safety and stability is that large pieces of meat may remain at a relatively high pH within the tissues, because the acids diffuse very slowly through solid foods. If this is not prevented, eventual equilibrium may be at a pH value higher than that of the freshly made product allowing a variety of organisms to grow. Marinating meat or fish (such as herring) to reduce the pH within pieces to a safe lower value can control such hazards. Proper chilling of a mayonnaise-based salad directly after production is important to provide protection against growth of spoilage organisms or pathogens when there are still parts of the salad at a near neutral pH.

Pathogens

Foodborne illness from mayonnaise-based salads can result from the presence of pathogens or toxins in the raw materials or from growth of pathogens in the salad. Table 30-3 gives a survey of some major outbreaks. The outbreak at the European Summit Conference in Maastricht[18] clearly was the result of pathogen multiplication as numbers of *Salmonella indiana* reached levels of 10^7 g^{-1}. In the report, however, reference is made to another cold buffet for which it is claimed that a low infective dose without significant multiplication of *S. indiana* caused illness of 19 persons. The relatively high mortality in two *Salmonella* outbreaks (see Table 30-3) may be explained by contamination with *S. typhi* in the first outbreak and a very sensitive elderly population (mean age 64.2 years) at risk in the last outbreak.

Pathogens other than *Salmonella* have been associated with foodborne illness from mayonnaise-based salads as shown by *E. coli* in a potato salad on a cruise ship (Table 30-3). *Shigella* is another significant source of illness to which its low infective dose may contribute. Smith refers to at least 11 outbreaks in the US in the period 1975–1981 with over 1,500 cases of illness due to *Shigella flexneri* or *S. sonnei* caused by various salads of which eight were potato salad.[115] The latter is striking and in line with Table 30-3, where potato salad was the vehicle of infection on five occasions. Ten further reports of staphylococcal food poisoning in the US due to potato salad are reported.[27] In fact a total of 41 outbreaks caused by potato salad is listed in this publication. This high rate of foodborne illness may be due to a relatively large proportion of neutral potato in these salads and a low level of acetic acid, permitting extended survival and growth of pathogens, particularly when temperature abuse takes place. *Listeria monocytogenes* contamination of coleslaw in Canada[111] is not referred to in the table as the coleslaw mix did not contain dressing.

Growth of pathogens represents an evident safety hazard for salads with a high pH, when salads are not held at refrigerated temperatures.[47] At 22° and 32°C *Salmonella* and *S. aureus* could grow well within 24 hours in chicken salad (pH 6.1) and ham salad (pH 5.2) but no growth occurred at 4°C. Kurihara et al. demonstrated rapid growth of *S. enteritidis* at 25°C in potato, egg, and crab salads for which the pH was 5.72, 7.11, and 6.51, respectively.[77] The salads were made with 15% of homemade mayonnaise (pH 4.75), which contained 0.1% of acetic acid. With an estimated dry matter level of 30% for the salads this re-

Table 30-3 Major Outbreaks of Foodborne Illness Due to Mayonnaise-Based Salads

Country	*Year*	*Salad Type*	*Production Facility*	*Pathogen/ Toxin*	*Probable Origin of Contamination*	*Cases*	*Fatal*	*Reference*
Germany	1974	Potato salad	Industry	*Salmonella* (Typhoid)	Contamination of potato and well water; defective chill chain	417	5	70
US	1974	Potato/tuna/ egg salad; coleslaw	Country fair	*Shigella sonnei*	Contamination of salad dressing; ambient holding of salads	248		130
US	1978	Potato salad	Restaurant	*C. botulinum* type A toxin	Toxin formation in baked potatoes due to temperature abuse	8		113
Netherlands	1981	Meat/ potato	Restaurant	*Salmonella indiana*	Cross-contaminated salad from contaminated kitchen	600–700		18
US	1984	Potato salad	Cruise ship	Invasive *E. coli*		47		118
US	1987	Tuna-macaroni	Hospital	*Salmonella enteritidis*	Mayonnaise made with raw egg; ambient storage of salad	404	9	121

sults in only 0.02% of total acetic acid in the aqueous phase of the salad and thus only a trace of undissociated acetic acid to prevent pathogen multiplication (0.002% at pH 5.7 and even less at the other pH values). Evidently pH, acetic acid level, and temperature in these tests were inadequate to prevent pathogen growth.

Similarly with *L. monocytogenes* in coleslaw at pH 6.0 growth was observed at 25°C, but not at pH 5.0.[58] At pH 5.7 significant growth of *Salmonella* and *L. monocytogenes* was seen in home-style chicken salad with mayonnaise at 12.8°C, but not at 4°C.[52] In macaroni salad at pH 4.6, no growth was observed.

The deduction from the above findings is that mayonnaise-based salads with a pH above 5.0 are likely to permit growth of pathogens when not kept under refrigerated conditions. The precise conditions to prevent pathogen multiplication, however, cannot be derived from these studies as many were done with salads at pH values above 5.0, where most of the acetic acid will be present in the inactive, dissociated form. Most home-style salads made with industrial mayonnaise will have sufficient antimicrobial properties to minimize health hazards according to Erickson et al.[52] and five factors are mentioned that contribute to this. Chilled storage and an inhibitory formulation are seen as the most important ones.

Infective pathogens introduced into a mayonnaise-based salad may be killed rapidly at low pH and high temperature.[69] However, pathogens may survive for days or weeks (and well beyond the salad's shelf life) under chilled conditions.[133] Contamination with infective pathogens thus presents a distinct safety hazard for the consumer.

30.5.5 Preservation

Of the weak food acids in mayonnaise-based salads, acetic acid is the most active with antimicrobial activity against lactobacilli and yeasts, while lactic acid shows good inhibition of lactobacilli but not of yeasts.[133] The pH of the mayonnaise for the salad can be set solely by the addition of acetic and/or lactic acid, but buffering with sodium hydroxide was proposed because this allows the addition of a higher percentage of these acids at a given pH and a better microbiological stability.[45] This approach proved to work particularly well for yeasts when acetic acid and lactic acid together were incorporated in a buffered salad at pH 4.85 to 4.95 with approximately 1% acetic acid and 1% lactic acid in the aqueous phase of the salad. Yeast growth was completely inhibited for 8 weeks at 6°C in such a salad.

Because of the commonly low levels of 0.2% to 0.5% of acetic (and/or lactic) acid in the aqueous phase of commercial mayonnaise-based salads these remain vulnerable to spoilage, and sorbic acid and benzoic acid are often used to improve stability. Within the EU a maximum of 1,500 mg kg^{-1} of either sorbic acid and/or benzoic acid is permitted (Table 30-2) and in practice often a combination of sorbic and benzoic acid is used. The chemical preservation is discussed by Wit and Rombouts,[133] who demonstrate that in oil-containing products there is a critical upper pH for benzoic acid (pH 4.9) and sorbic acid (pH 5.1), above which there is no increase in effective undissociated acid because of the distribution coefficient water/oil of the preservatives and the pKa of the acids. When preservative-resistant yeasts are present the legally permitted maximum still may be inadequate as shown by spoilage of a chilled herring salad of pH 4.0 with 500 mg kg^{-1} of sorbic acid and 1,100 mg kg^{-1} of benzoic acid, which was spoiled by *Zygosaccharomyces rouxii*.[16] Depending on the initial contamination of the salad, the pH, presence of nutrients (sugars), levels of inhibitory food acids and levels and types of preservatives (if any), the typical shelf life limited by spoilage or quality loss varies from 2 to 8 weeks under chilled conditions.

Normally, mayonnaise-based salads do not permit growth of pathogens because of the acetic acid in the aqueous phase, the reduced pH, and chilled storage.[52,133] In salads with very little acetic acid and a pH above 5.0, however, pathogens may multiply, especially if the chilled chain is interrupted. The Dutch Industrial Code for Salads refers to a pH of max. 5.0, a storage temperature below 7°C, and the presence of 0.3% acid (of which 0.2% should be acetic acid), as design criteria to prevent growth of any pathogens.[12] With an assumed moisture content of 70% and 36% of the acetic acid present in the undissociated form at pH 5.0, this corresponds to $0.2 \times (100/70) \times (36/100) = 0.10\%$ undissociated acetic acid in the aqueous phase of the product, which will control pathogens under chilled conditions. Preservatives can be used in addition to the above factors, but these will contribute more to stability than to safety.

The German "Feinkostindustrie" recommends for mayonnaise-based salads a temperature below 7°C, a pH of ≤ 4.6, and 0.2% acetic acid in the aqueous phase of the product, which corresponds to *ca.* 0.1% undissociated acetic acid. Chilled storage is considered particularly important as directly after production some components may still have near neutral internal pH values. For salads where such a low pH is not well compatible with the raw materials used (shellfish) 0.3% acetic acid in the aqueous phase and a pH ≤ 5.0 is proposed, which also corresponds with 0.1% undissociated acetic acid. Evidently, safe chilled salads may be made at higher pH values with different preservation concepts (pasteurization, reduced water activity, natural preservatives, short shelf life), but a HACCP-based approach should be followed to prevent pathogen hazards.

A new approach for preservation of sauce-based delicatessen salads was followed by Bonestroo, who stabilized salads by in-pack fermentation with starter cultures of lactic acid bacteria at temperatures ≥ 42°C for a period of ≤ 8 hours, followed by cooling to below 7°C. In this way a shelf life of 5 to 6 weeks at 7°C proved possible.[22] During fermentation some increase (< 1 log) of *L. monocytogenes* and *S. aureus* was noticed, followed by a rapid decline in numbers (*ca.* 2 log).

Control over safety and spoilage hazards of mayonnaise-based salads can be summarized as a step program, which is largely based on van Schothorst[112]:

1. Determine in the design phase whether any pathogens can grow under the intended (chilled) storage conditions in the mayonnaise-based salad and select a formulation that will prevent this.
2. Determine carefully the microbiological status of all ingredients and do not use ingredients that present uncontrolled infectious pathogen hazards.
3. Use cooked, blanched, or pasteurized ingredients whenever feasible.
4. Clean and wash raw vegetables to remove all adhering dirt.
5 Dice precooked meats and poultry into small pieces, and/or marinate them in acid before mixing them with other ingredients.
6. Keep the pH as low as possible (while retaining good eating quality) by the use of sauces and dressings containing organic acids (usually acetic acid from vinegar).
7. Use strict hygiene in preparing, mixing, and storing the salads.
8. Establish a realistic shelf life for the salad by holding product slightly above the intended chilled temperatures during storage and use. For salads sold at 7°C, for example, this would mean holding at 10°C, for example, for a number of weeks followed by visual inspection (no blowing, no visible deterioration or spoilage), pH measurement (no drop in pH), organoleptic evaluation (good preservation of taste and appearance), and microbiological analysis (no excessive counts of yeasts or lactobacilli). The shelf life established at 10°C is then used at 7°C.
9. Perform a HACCP study of the manufacturing process, from raw material to consumer use, to check whether the actual product and process is in line with all the requirements that were considered critical in the design phase.

30.6 PASTEURIZED ACID SAUCES

A range of acid or acidified sauces exists, normally not acidified with vinegar or acetic acid, which are preserved by heat processing and a pH of max. 4.6. High water activity, ambient-stable sauces with a pH above 4.6 without intrinsic stability factors are low-acid foods, which are preserved by sterilization.[34] These are not considered in this section. Many of the pasteurized acid or acidified sauces are tomato-based and are sometimes referred to as pasta sauces (tomato sauces for pasta meals) or cook-in sauces for chicken and meat or stir and fry sauces for meat and vegetables. White sauces exist also. They all have in common that they are used on a host food. Because of the low pH and heat processing, the key target organisms to be controlled are acid-tolerant, spore-forming bacteria. The sauces have a long closed shelf life of a year or more, with a short open shelf life of a few days under refrigeration.

30.6.1 Composition

The composition of the sauces often includes fresh or processed tomatoes. The latter are used as canned whole, pieces, diced, or sliced tomato or as tomato puree or paste. Commonly used acidulants are citric, lactic, and malic acid and sometimes also acetic acid and phosphoric acid. Herbs and spices are used as in mayonnaise with thickeners like starches and gums. Further ingredients can be sugar, salt, condiments, flavors, and pieces of vegetables, fruits, and also meat or cheese.

30.6.2 Initial Microflora

The initial microflora of the ingredients includes those already mentioned in Section 30.2.2 for mayonnaise, but the vegetative bacteria, yeasts, or molds are easily inactivated during heat processing and not significant for product safety or stability. The ingredients like spices, herbs, tomato, and vegetables will carry a significant bacterial spore load, of which in particular the acid-tolerant types are relevant. One of the more significant ones is *Bacillus coagulans*, which is present in tomato, vegetables, dairy raw materials, and herbs and spices. For example, in whole, peeled tomatoes, sampled before canning, median values of 18 to 40 spores ml^{-1} were found in samples of three canneries.[134]

30.6.3 Effect of Processing

The acid sauces receive a heat treatment, which typically is given during processing, followed by a hot fill, and a postpasteurization process for filled containers. The heat treatment is intended to inactivate all infectious pathogens and non–spore-forming microorganisms. The acid-tolerant spore-formers, which may be able to grow in the product, either should be killed by the heat process or heat damaged to such an extent that they will not recover and grow in the product. The heat process is typically the equivalent of 10 to 30 minutes at 90° to 100°C, although milder and more severe processes exist. The latter would be the case for acid sauces with relatively high pH (close to 4.6), while much milder processes may be applied for sauces with a pH close to 4.0–4.2.

30.6.4 Spoilage Associations and Pathogen Hazards

The main spoilage organism of concern for acid sauces is *Bacillus coagulans*, formerly called *Bacillus thermoacidurans*.[20] The latter name refers to the preference for elevated temperatures and tolerance to acid conditions. *Bacillus coagulans* is strongly associated with tomato products, but may be found also in other raw materials like vegetables, spices, or dairy products. *B. coagulans* causes flat-sour spoilage, ie, acid formation and no gas, when the spores germinate and grow in acid sauces. The resultant drop in pH typically is small (about half a pH unit) but may also be nil,[108,134] and can be accompanied by a typical taste. Because little information has been published on spoilage of acid sauces, many of the preservation processes are derived from those applied for tomato and other acid or acidified products. An interesting example is a study on spoilage of canned, whole peeled tomatoes in juice for which in the 1960s a heat process equivalent with 0.7 minutes at 121.1°C was proposed by the National Canners Association (NCA) to control *B. coagulans* in products with a pH of 4.5, when acidification with citric acid was not yet permitted.[95] It was later established that spores of *B. coagulans* did grow well in tomato juice, acidified with citric acid to pH 4.45, when heated for 10 minutes at 90°C, but no growth occurred when the pH was reduced to 4.31.[134] Without any heat, the spores could germinate and grow at pH 3.8 when the inoculum was sufficiently high with 640 spores ml^{-1}. The combination of mild heat and reduction of pH to 4.3 apparently caused sufficient heat damage of the spores to ensure product stability under conditions that were well below those recommended earlier by the NCA. The feasibility of the reduced heat process was documented in a process for peeled tomatoes equivalent to 1.56 minutes at 100°C, which did not give any spoilage when challenged with spores of *B. coagulans* ATCC 8038.

Spoilage of tomato products may also be caused by butyric anaerobes such *as Clostridium pasteurianum* or *C. butyricum*. This is a rarer type of spoilage, first described for tomato juice, and causing a bad butyric smell of the juice. Bowen et al.[23] studied spoilage of canned tomatoes and showed that acidification with 0.1% citric acid to pH 4.3–4.4 and heating to a can center temperature of 200°F (93.3°C) was adequate to control contamination with spores of *C. pasteurianum*. This finding is in line with the NCA published Thermal Death Curve for *C. pasteurianum* with 10 minutes at 200°F at pH 4.5.[93] An alternative way to achieve stability was acidification with 0.2% citric acid to a pH of 4.1 or less and heating to 90°C.[23]

Leaker spoilage by lactobacilli or yeasts may occur when cans or jars are not hermetically closed, or when heat-resistant molds survive the heat process. This may introduce a safety hazard because of a local increase in pH and an unexpected botulinum hazard.[66] Otherwise neither *B. coagulans* nor other spoilage organisms of acid sauces present a hazard.

30.6.5 Preservation

Clostridium pasteurianum is less heat resistant than *B. coagulans*; heat processing of pureed tomatoes at pH 4.3 to 4.4 to a center temperature of 200°F (=93.3°C) prevented spoilage.[23] The NCA indicated that a process equivalent to 10 minutes 93.3°C would be adequate for butyric anaerobes in products with a pH above 4.3 and 5 minutes when the pH was between 4.0 and 4.3.[95] Other sources refer to a product temperature of 95°C when the pH is between 4.2 and 4.5, whereas heating to 84°C may be adequate at a pH below 4.2. For acid sauces this spore-forming organism normally is of little significance as the heat process required to control *B. coagulans* is well above that for *C. pasteurianum*.

Bacillus coagulans spores are rather variable in heat resistance. Published D-values in tomato puree at a pH of 4.3–4.5 range from 9.0 to 12.3 minutes at 90°C[108] to 0.23 minutes at 121°C.[135] Also Put and Wiebenga[102] found a high heat resistance for spores of *B. coagulans* strain Sp. 33 in phosphate buffer of pH 6.8 with a D_{110} of 2.4 minutes. Even a D_{121} of 3 minutes was reported.[93] The process proposed[108] for crushed tomato at pH 4.5 was 2.56 minutes at 110°C. The FDA recommendation for products at this pH is 310 to 500 minutes at 95°C (equals *ca.* 10 to 15 minutes at 110°C), which is a more severe process.

The heat resistance of other acid-tolerant sporeformers in tomato juice was investigated[109,110] because there was concern that growth of these *Bacilli* may raise the pH of the juice and permit spores of *Clostridium botulinum* to grow and produce toxin. The D_{100} for *Bacillus subtilis* was 5.7 minutes and for *B. licheniformis* 5.9 minutes, with z-values of 14° and 14.2°C. Under aerobic conditions, both *Bacillus* species could grow in juice at pH 4.4 and raise the pH to 4.8. Under anaerobic conditions both organisms could not germinate and outgrow in juice at pH 4.4. It was concluded that spores, which may survive the commercial heat process for tomato juice of 10 to 60 minutes at 100°C, will only grow if aerobic conditions are present in the can.[110] Based on these findings, both *Bacillus* spp. may not be very relevant as spoilage organisms for acid sauces, although *B. licheniformis* is considered important for acidified canned vegetables.[97]

The key issue in many existing processes to control *B. coagulans* in acid sauces is that it is not always clear whether the proposed process is aimed at inactivation of all spores (which will require very severe processes) or will inactivate some spores and damage the rest to prevent them from germination and growth in the product because of its reduced pH and the natural weak acids present. A further aspect to consider is that not all products will be exposed to temperatures that allow spoilage. Temperatures of 35° to 45°C are optimal for *B. coagulans* growth in acid sauces, but in temperate climates these temperatures will be rare. Prompt cooling after heat processing can prevent the occurrence of such temperatures. The following example may demonstrate the influence of storage temperature.[15]

A tomato-based pizza sauce with a pH of 4.5, a_w of 0.98, and 500 mg kg^{-1} of sorbic acid was preserved by heating to 95°C for 2 to 5 minutes followed by hot fill at 80°C in aluminum foil pouches and a postfilling pasteurization for a few minutes at 85°C. This sauce had a documented shelf life of 6 months at ambient temperatures of 25°C with no evidence of spoilage. When samples of the same sauce were stored at 37°C they spoiled within 2 weeks because of growth of *B. coagulans*. A more detailed study showed an initial load of *B. coagulans* spores of 50 to 100 g^{-1} of the sauce gave no spoilage at all within 24 weeks at 15°, 20°, 25°, 50°, and 55°C. At 37° and 42°C *B. coagulans* caused a pH drop to 3.9 and spoilage of the sauce within 10 days. At 30°C, spoilage was noticed after 4 to 6 weeks and at 46°C after 2 weeks.[15] The example shows that the spoilage of such an acid sauce can be strongly temperature dependent and a sauce that may be stable in the temperate conditions could spoil when distributed at warmer Mediterranean conditions. For the latter case, heat processing to a temperature above 100°C may have been required.

Thus, for acid sauces, a range of heat processes may be applied, ranging from a few minutes at 90°C to well over 110°C depending on inactivation or heat damage of acid-tolerant spores present. For development of any new process, carefully establishing the impact of product composition, pH, weak acids, initial spore load, the overall heat process, and intended storage temperatures on the control of the target organism is required. In some countries the use of preservatives (sorbic or benzoic acid) is permitted, which may further contribute to stability. Good understanding of these factors may result in significantly reduced heat processes and better product quality. Care should be taken for sauces with neutral meat, vegetable, or cheese particulates to ensure that these particulates are properly adjusted to a pH value below 4.6; otherwise, a botulinum hazard may be created in pockets with elevated pH. For pH adjustment, the type of acid may not be not very significant because at pH 4.5 no difference was noted in inactivation of *B. coagulans* spores at 105° or 110°C after acidification with lactic, citric, acetic, malic, or hydrochloric acid. With further reduction to pH 4.2, this changed and acetic and lactic acid gave faster inactivation.[86]

30.7 CONCLUDING REMARKS

The products discussed in this chapter are characterized by a reduced pH, often achieved with weak acids such as acetic acid or lactic acid. This combination has given industrial products significant protection against pathogen growth and foodborne illness, but the use of contaminated raw materials, such as raw egg and/or inadequate control over equilibrium pH of final products has permitted major outbreaks of food poisoning. Currently, the safety aspects of these products are well understood and the importance of using pasteurized egg in all emulsified products is generally recognized by food hygiene professionals, whether products are industrially prepared or made in large kitchens. The application of HACCP will add to the product safety of all these products and risk analysis techniques as applied by Whiting and Buchanan[132] for the *Salmonella enteritidis* hazard of homemade mayonnaise will give even better understanding of the significance of the key factors to product safety. In view of the preference for milder and less processed products there is, however, still a need to establish more accurately safe combinations of pH and undissociated, weak acids for emulsified and nonemulsified dressings and sauces. Otherwise, it may occur that acetic acid in a formulation is replaced with lactic, citric, or another weak food acid, without due consideration of loss of antimicrobial activity associated particularly with undissociated acetic acid. When combined with an increase in pH to values of 4.5 and above, such change may lead to loss of intrinsic stability and recurrence of known product safety problems. Therefore, within our company a microbiological expert system, MIDAS (Microbiological Design Approval System), was developed, which assists companies in developing dressings that are safe because of an integrated evaluation of raw material, processing, and shelf life requirements, resulting in an approved safe design.[42]

With respect to product stability, there will be options for application of novel preservation technologies, such as natural antimicrobials and high pressure. For an up-to-date overview of these new technologies, the reader is referred to *New Methods of Food Preservation.*[64] Nisin inhibits most lactic acid bacteria in beer,[96] and its application for inhibition of spoilage lactobacilli in dressings was reported.[92] Nisaplin at 200 mg kg^{-1} (equivalent to 5 mg kg^{-1} of nisin) gave full inhibition of 10^4 g^{-1} *Lactobacillus brevis* subsp. *lindneri* inoculated into ranch dressing with a pH of 3.8, 4.0, and 4.2 at 26°C. In the dressing at pH 4.2 without nisin, the inoculated lactobacilli grew in 30 days to 10^8 g^{-1}. For dressings, the use of nisin will be granted in the US to inhibit lactobacilli, although the price may delay its introduction on a commercial scale.

Nisin was also studied for inhibition of *B. coagulans*, and a level of 1 IU ml^{-1} nutrient agar gave inhibition of 10^3 spores per plate. Combinations of high hydrostatic pressure, reduced pH, and nisin gave further inactivation of spores. A 6 log reduction was achieved at 70°C with a pressure of 400 Mpa in pH 4.0 buffer for 30 minutes, when plated in nutrient agar with 0.8 IU nisin.[107]

Natural antimicrobials from extra virgin olive oil may present an additional option to control pathogens in oil-based sauces.[103]

It is expected that a better understanding of the traditional preservation processes and the application of novel preservation technologies will allow the development of further new and safe products. Much care must be taken in this process to prevent new preservation options from providing new ways of transmitting foodborne illness, as more natural may not necessarily mean more safe.

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31

Fruit Juices, Fruit Drinks, and Soft Drinks

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31.1 INTRODUCTION

Over the past two decades, the soft drink industry has been booming,[45,188,207] with annual growth approaching 10%. Production by volume of soft drinks, including mineral waters, has been predicted to reach 50bn liters in 1997.[93] Expansion has been global,[207] growth being particularly strong in the less-developed markets of Southeast Asia.[45] Carbonated beverages, despite rising consumption of fruit juices, still account for about 50% of the market.

Current trends in the mature markets of the developed world are toward healthier "back-to-nature" styles of soft drink,[188] including a proliferation of "New Age" drinks, sports drinks, fiber drinks, flavored ice teas, and fruit-flavored mineral waters that blur the traditional distinctions between beverages and exotic fruit drinks. Demand for traditional fruits is likely to outstrip supply,[188,189] leading to increased use of more exotic fruit juices. The market for fruit juices is currently dominated by Europe and the United States (US). Five major markets, US, Germany, United Kingdom (UK), Netherlands, and France, account for 65% of total world imports,[188] the majority of this trade being in frozen concentrated orange juice (FCOJ). Orange represents 59% to 73% of US imports, followed by apple/pear 16% to 24%, and pineapple 7%.[188]

Much has been written concerning the microbiology of soft drinks and fruit juices. Knowledge of likely spoilage organisms in these products has been widely disseminated, both within the industry and in academic circles.[21,50,176,204,282] Yeasts, such as *Zygosaccharomyces bailii*, remain the principal organisms of contention because of their physiology and phenomenal resistance to chemical preservatives.[130]

However, the technology of soft drinks manufacture has progressed, with possible new preservation technologies including ultra high pressure, irradiation by ultraviolet or optical wavelengths, processing in sterile environments and heat treatments; the

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We wish to gratefully acknowledge the help and advice of Professor Bob Davenport in preparing this review, particularly concerning the spoilage or hygiene role of contaminant yeasts.

ultimate goal being fast, low cost, aseptic filling. There has also been a proliferation of new packaging materials and formats, including plastics, metal-foil, and cardboard laminates.

Despite modern technology, spoilage of soft drinks and fruit juices continues to occur. While microorganisms and raw materials remain unaltered, manufacturing practice is undergoing change motivated by increasing competition, new technology, and public demands for more "natural" products.[93] New technology itself has created new microbiological problems; an example being the plastic PET (polyethylene tetraphthalate) bottle. This is an attractive, light, and convenient packaging format, able to contain carbonated products, and very popular with consumers.[198,249] However, it has the twin disadvantages of allowing oxygen permeation through the plastic and of not being able to withstand pasteurization temperatures without distortion, thus requiring aseptic filling or addition of preservatives.

Throughout the developed world, the public is becoming more aware of the presence of chemical residues in foods. This may have arisen from agricultural overuse of biocides that persist in the food chain, but has had the result of inducing public antagonism to all perceived chemical adulteration of foods. In parallel has come approval and demand for more herbal and "natural" products and processes.[93] Legislation has, in general, reflected public concern and political necessity rather than science, and has moved toward removal of preservatives and to reduction in permitted levels. Preservative legislation varies widely across the world. In the European Community (EC), current limits in soft drinks are 300 ppm sorbic acid or 150 ppm benzoic acid,[14] whereas in the US these preservatives have generally recognized as safe (GRAS) status and their concentration is, therefore, only limited by taste considerations. One of the most significant spoilage organisms of soft drinks and fruit juices is the yeast *Zygosaccharomyces bailii,* which can grow in excess of 500 ppm sorbic acid. Permitted concentrations of preservatives are already so low as to render some products microbiologically unstable. Such public and legislative trends are likely to result in increased incidents of microbial spoilage.[20]

31.2 COMPOSITION AND CHARACTERISTICS OF FRUIT JUICES AND SOFT DRINKS

31.2.1 Definitions

Fruit Juices

Fruit juices are the unconcentrated liquids extracted, usually by pressure, from mature fruit. Legal definitions of fruit juices, permitted additives, and treatments vary between countries and are reviewed by Pollard.[206] The EC Directive states that fruit juices are the juices extracted from fruit by mechanical processes, fermentable but unfermented, having the characteristic odor, color, and flavor typical of the fruit from which they come.[206] Pectolytic enzymes may be added to certain fruits, such as raspberries, blackcurrents, strawberries, and plums, prior to pressing, to break up the cell structure and enable free flow of juice.[79,120] Pressed juices often contain large amounts of insoluble solid material and may require clarification. Ascorbic acid (vitamin C) may be added to juices (Figure 31-1) as an antioxidant.[206] Juices may be sold fresh, concentrated by evaporation, preserved by pasteurization or more rarely, in the case of grape juice, preserved at low temperature with sulfites.[269]

Fruit Juice Concentrates

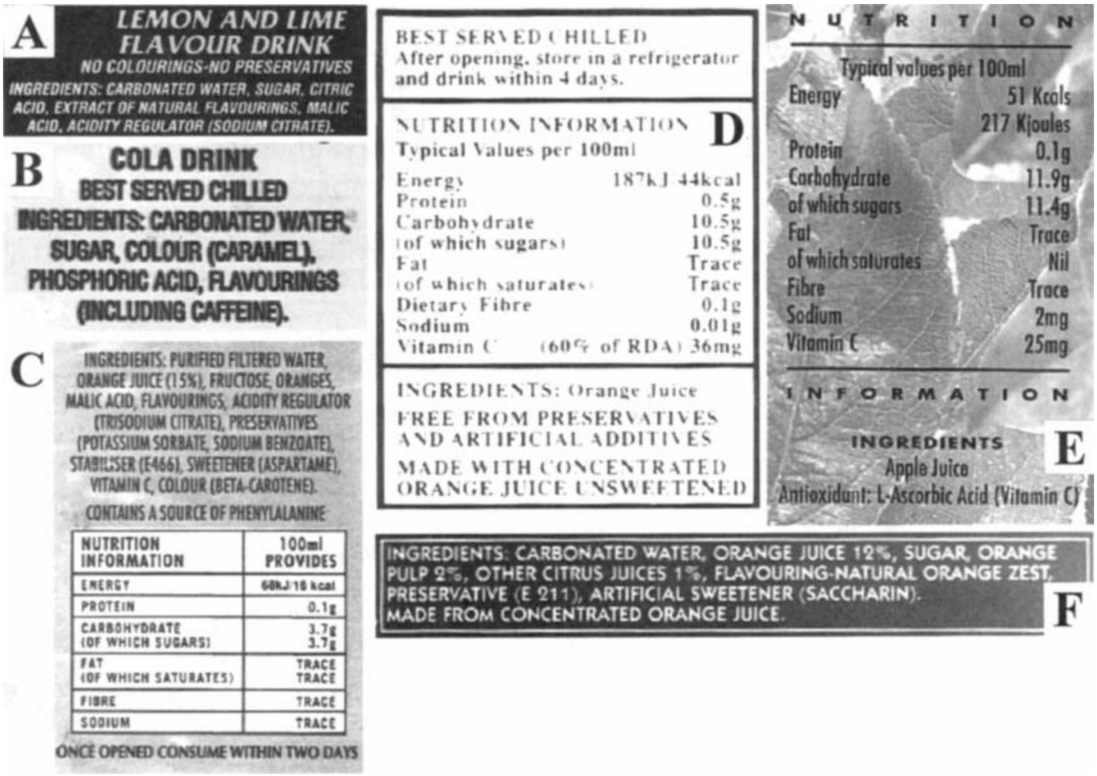
Concentrates are prepared from juices by water evaporation under vacuum at elevated temperatures. Volatiles such as citrus essences are recovered by fractionation and blended back into concentrates.[214] Obvious advantages of concentration are volume reduction in storage and shipping and microbiological stability if sufficiently concentrated and chilled. Much of the world trade in fruit juices involves concentrates, notably frozen concentrated orange juice (FCOJ).[133]

Fruit Nectars

Nectars are partially diluted pulpy liquids prepared from juices or concentrates. The EC Directive[206] considers nectars to be unfermented but fermentable products, obtained by the addition of water and sugar to fruit juice, concentrated fruit juice, or fruit puree. Many fruits juices are highly acidic and considered inedible in their natural state, eg, juices from sloes, passionfruit, or gooseberries, and their nectars must contain a minimum of 25% juice. Nectars from less acidic, edible juices, such as citrus or apples, must contain a minimum of 50% juice.

Soft Drinks

Soft drinks is a term encompassing a wide variety of products, fruit-based or otherwise, carbonated or still. This diversity has caused regulations concerning soft drinks to be more concerned with permitted additives rather than defined contents. Still soft drinks are predominantly fruit based and include fruit drinks, fruit juice drinks, and fruit squashes. More recently, a number of still tea-based beverages have been marketed. These range from products



LEMON AND LIME FLAVOUR DRINK
NO COLOURINGS-NO PRESERVATIVES
INGREDIENTS: CARBONATED WATER, SUGAR, CITRIC ACID, EXTRACT OF NATURAL FLAVOURINGS, MALIC ACID, ACIDITY REGULATOR (SODIUM CITRATE).

COLA DRINK
BEST SERVED CHILLED
INGREDIENTS: CARBONATED WATER, SUGAR, COLOUR (CARAMEL), PHOSPHORIC ACID, FLAVOURINGS (INCLUDING CAFFEINE).

INGREDIENTS: PURIFIED FILTERED WATER, ORANGE JUICE (15%), FRUCTOSE, ORANGES, MALIC ACID, FLAVOURINGS, ACIDITY REGULATOR (TRISODIUM CITRATE), PRESERVATIVES (POTASSIUM SORBATE, SODIUM BENZOATE), STABILISER (E466), SWEETENER (ASPARTAME), VITAMIN C, COLOUR (BETA-CAROTENE).
CONTAINS A SOURCE OF PHENYLALANINE

NUTRITION INFORMATION	100ml PROVIDES
ENERGY	68kJ/16 kcal
PROTEIN	0.1g
CARBOHYDRATE (OF WHICH SUGARS)	3.7g 3.7g
FAT (OF WHICH SATURATES)	TRACE TRACE
FIBRE	TRACE
SODIUM	TRACE

ONCE OPENED CONSUME WITHIN TWO DAYS

BEST SERVED CHILLED
After opening, store in a refrigerator and drink within 4 days.

NUTRITION INFORMATION
Typical Values per 100ml

Energy	187kJ 44kcal
Protein	0.5g
Carbohydrate	10.5g
(of which sugars)	10.5g
Fat	Trace
(of which saturates)	Trace
Dietary Fibre	0.1g
Sodium	0.01g
Vitamin C	(60% of RDA) 36mg

INGREDIENTS: Orange Juice
FREE FROM PRESERVATIVES AND ARTIFICIAL ADDITIVES
MADE WITH CONCENTRATED ORANGE JUICE UNSWEETENED

NUTRITION
Typical values per 100ml

Energy	51 Kcals 217 Kjoules
Protein	0.1g
Carbohydrate	11.9g
of which sugars	11.4g
Fat	Trace
of which saturates	Nil
Fibre	Trace
Sodium	2mg
Vitamin C	25mg

INFORMATION
INGREDIENTS
Apple Juice
Antioxidant: L-Ascorbic Acid (Vitamin C)

INGREDIENTS: CARBONATED WATER, ORANGE JUICE 12%, SUGAR, ORANGE PULP 2%, OTHER CITRUS JUICES 1%, FLAVOURING-NATURAL ORANGE ZEST, PRESERVATIVE (E 211), ARTIFICIAL SWEETENER (SACCHARIN). MADE FROM CONCENTRATED ORANGE JUICE.

Figure 31-1 Typical Examples of Content of Soft Drinks. **A**, carbonated fruit drink containing citric and malic acids, preserved by pasteurization; **B**, carbonated cola-type soft drink containing phosphoric acid; **C**, fruit drink preserved with benzoic and sorbic acids; **D**, fruit juice prepared from a concentrate and pasteurized; **E**, fruit juice containing ascorbic acid as an antioxidant; **F**, carbonated fruit-containing soft drink, preserved with benzoic acid.

formed from direct leaf extraction to those made from spray-dried tea powders. Ready-to-drink teas are slightly sweetened and are typically flavored with lemon or other fruits.

Carbonated Soft Drinks

This category includes colas, sparkling fruit drinks, mixers such as tonic or ginger ale, lemonade, carbonated teas, shandy, and cream soda. Carbonation involves dissolving carbon dioxide in the beverage under slight pressure, forming sparkling beverages. It is measured in Volumes Bunsen, CO_2 volumes at 0°C and 1 atm, dissolved per volume liquid, or in grams CO_2 dissolved l^{-1} ($1.96\ g\ l^{-1} = 1$ volume[172]). Carbonation of soft drinks is typically around 3 volumes, varying from 1.5 in sparkling orange to 5 in soda water. Low carbonation can be achieved by carbonate addition but more usually by pressurized CO_2 addition during bottling.[279] Soft drinks may also contain additions of sugars, vitamins, acids and their salts as acidulants and acidity regulators, flavorings, artificial sweeteners, colorings, emulsifiers and stabilizers, antioxidants, and chemical preservatives (Figure 31-1). Additives and their concentrations vary widely depending on legislation pertaining to the area of sale or production.

Alcoholic "Soft" Drinks

Alcholic "soft" drinks were launched on the UK market in 1994–1995. Alcoholic soft drinks typically contain 4% to 6% ethanol in a fruit-based soft drink. The majority are not fermented products, and their microbiology resembles that of soft drinks.

31.2.2 Chemical Composition

Fruit juices and soft drinks are essentially aqueous chemical solutions, sometimes containing a few percent suspended solids. These range from the simplicity of a lemonade, carbonated water, sugar, and citric acid, to the complexity of mixed fruit juices. Characteristic of all soft drinks and fruit juices are low pH and a fermentable sugar content (zero-sugar drinks excepted).

pH, Buffering Capacity, and Titratable Acidity

pH is the negative log of the hydrogen ion concentration. pH indicates only the free H^+ concentration and does not indicate the size of the proton reservoir held combined by buffering. Buffering capacity is a measure of the ability of a solution to resist change of pH. In soft drinks, buffering capacity indicates the capacity of a beverage to maintain pH value, against changes induced by microbes (Figure 31-2). Titratable acidity measures the amount of alkali needed to titrate the acids present and is usually expressed as equivalents of citric acid in g l^{-1}. Almost all buffering in soft drinks and fruit juices is due to organic acids and acidulants.

In soft drinks, the taste threshold for acidity is in the region of pH 3.7–4.1,[106] sourness increasing with lower pH. However, perception of sourness is more closely linked with titratable acidity and buffering capacity than absolute pH value. The pH of most fruit juices (Table 31-1) ranges from 2.5–4.0,[119] limes being exceptional at pH 1.6–3.2 and pineapple averaging pH 3.8–4.0.[122] After long storage, the pH of apples may rise to pH 5.0.[271] Soft drinks have pH values largely in the range 2.5–3.0, except for diet/light formulations, pH 3.2–3.5. Tea-based beverages pH values tend to be slightly higher, pH 3.2–4.2.

Sugars

Fruits are designed to be eaten, appealing in color, scent, and sweetness. Hexose sugars constitute the largest soluble fraction of fruit juices. Fruit juices contain *ca.* 10% sugar (Table 31-1). Strawberries and raspberries contain less while grape juices contain up to 25% sugar. The perceived sweetness of fruit juices is modified by presence of acids and the sugar type, fructose tasting sweeter than glucose, for example.

The principle sugars in fruit juices are fructose, glucose, and sucrose. Proportions of sugars vary with fruit variety and ripeness (Table 31-1). Apples contain a higher proportion of fructose,[148] traces of xylose,[308] and moderate amounts of sorbitol, about 4 g l^{-1}, in contrast to pears, which contain up to 20 g l^{-1} sorbitol.[277] Grape juices contain more glucose, particularly at earlier stages of ripeness,[307] traces of pentoses,[6] and negligible sucrose. Apricot, peach, and pineapple juices are particularly rich in sucrose.[267] Changes occur in sugar composition during storage, notably the inversion of sucrose to glucose and fructose.[28] Up to 15 g l^{-1} sugar may be added to fruit juices for modification of perceived acidity.[206]

Soft drinks contain sugars at similar concentrations to fruit juices, typically ranging from 7% to 12%.[279] Concentration of sugar in soft drinks is customarily measured in degrees Brix, the w/w percent-

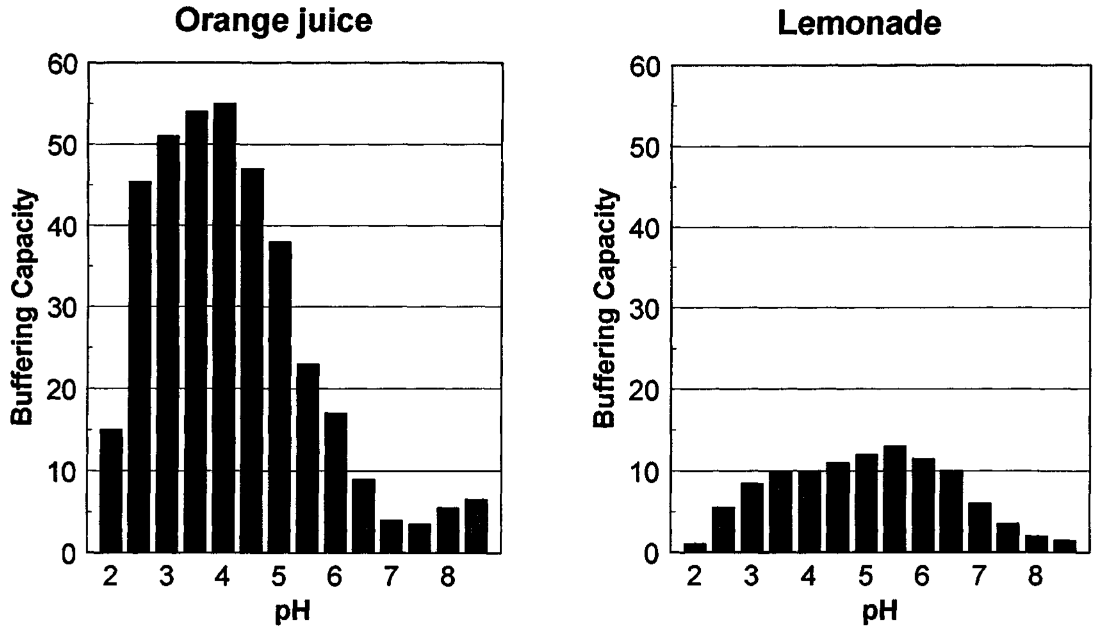


Figure 31-2 Buffering Capacity of a Fruit Juice, Orange, and a Soft Drink, Lemonade. Samples were de-carbonated, the pH was raised to 9.0 with NaOH, and titrated with 0.1 M HCl. One unit of buffering capacity is the amount of protons (m moles) required to change the pH of 1 liter by one pH unit.

Table 31-1 Sugar Content, pH Range, and Major Acids Present in Various Fruits

Fruit	*Sugar (w/w%)*	*pH Range*	*Major Acids*
Apple	10–14	2.9–4.5	Malic, citric
Apricot	6–7	3.1–4.5	
Cherry	9–13	3.7–4.4	Malic citric
Grape	15–25	2.9–3.9	Tartaric, malic
Grapefruit	8.8–10.2	2.9–3.6	Citric
Guava	9	3.2–4.2	Citric, malic
Kiwifruit	12	*ca.* 4.0	
Lemon	8–9	2.0–2.55	Citric
Lime	9	1.6–3.2	Citric
Mango	15–17	*ca.* 4.35	Citric, tartaric
Orange	9–12	2.6–4.3	Citric, malic
Passionfruit	15	2.6–3.4	Citric, malic
Peach	8–9	3.6–4.0	Malic, citric
Pear	9–11	3.0–4.5	Malic, citric
Pineapple	12–15	3.1–4.0	Citric, malic
Plum	9–11	3.0–4.5	Malic, quinic
Raspberry	6–7	2.5–3.1	Citric
Strawberry	5–6	3.0–3.4	Citric

Source: Data from 21, 47, 92, 122, 144, 148, 267, 268

age of the sugar concentration in solution. Sugars may be added in dry granulated form (sucrose) or as sugar syrups, usually 67° Brix. These can be of sucrose, glucose-type syrup produced from maize, or high-fructose glucose syrup produced from wheat.[29] Diet formulations of soft drinks contain less sugar (*ca.* 1%), the sweetness balance being made up with artificial sweeteners.

Acids, Acidulants, and Acidity Regulators

The characteristic taste of a soft drink is imparted by a balance of sweetness, from sugars, with acidity from various organic acids. After sugars, acids constitute the second largest constituent of fruit juices and soft drinks. Acids comprise various organic acids from fruit juices and acidulants. Acidulants are acids added to lower the pH; acidity regulators are the salts of these acids, added to increase the pH. The result is a solution of defined pH, containing organic acids and their salts, the proportions depending on the pH value. Concentrations and types of acids present in juices are characteristic of the fruit. Malic acid predominates in apples, cherries, and plums; citric acid in citrus fruits, such as orange, lemon, grapefruit, and in black currants and strawberries; and tartaric acid is unusual and is characteristic of grape juices. Sizable fractions of other acids may be present, such as quinic acid in apples. Ascorbic acid is also present. The concentrations of acids in fruit juices are usually of the order of 1%.[103] Malic acid in apples can range from 0.18% to 1.4%.[148] Berry fruits tend to be higher in acidity, notably black currant containing 4% citric acid.[99]

Acidulants added to soft drinks are often identical to those in fruit juices; citric, malic, and tartaric acids predominating. Different acids have different taste characteristics, such as the light, fruity character of citric acid, a fuller, smoother flavor for malic acid, and a sharper flavor for tartaric acid.[279] Other acids used are phosphoric acid in cola-type soft drinks, lactic acid for a smooth flavor, acetic acid in nonfruit drinks, fumaric acid, and adipic acid, notably in the US market.[265,279] Amounts used in soft drinks, in citric acid equivalents of titratable acidity, are of the order of 1–4 g l^{-1}.

Nitrogen-Containing Compounds

Fruit juices in general contain low concentrations of nitrogen-containing compounds: ammonium ions, amino acids, and peptide fragments. Orange juice contains 3–4 g l^{-1} amino acids, the majority of this being proline. Ammonium ions are seldom present at more than 25 mg l^{-1}.[103] Apple juices contain about 1 g l^{-1} amino acids, the majority being asparagine, with lower amounts of aspartic acid and glutamic acid.[17,43] Grape juices are variable in nitrogen content, ranging from 90–840 mg l^{-1}, as equivalents of ammonium ions.[174] Grape nitrogen content was directly correlated with nitrogen in the soil and application of fertilizer.[30,199] Amerine and Ough[5] examined 29 grape juices that averaged 440 mg l^{-1}. Some 80% of this comprised amino acids, although proline again predominated.[123] Soft drinks contain nitrogen-containing compounds derived from fruit components, and from organic flavorings. The artificial sweetener, aspartame, can break down to yield phenylalanine.

Phosphates

Phosphates are present in relatively low concentration in most soft drinks and fruit juices, the notable exception is colas acidified with phosphoric acid. Phosphorus contents of 110, 210, and 180 mg kg^{-1} have been reported in apples, oranges, and peaches, respectively.[84] Higher phosphate levels have been reported in grape musts, 200–500 mg l^{-1},[4] variations are because of fruit variety, ripeness, growing region, and fertilization practices.[107,144]

Salts and Trace Elements

Fruit juices are characteristically rich in potassium but contain relatively low concentrations of sodium, calcium, magnesium, iron, copper, and zinc, compared with whole fruits. This is due to the adsorption of inorganic nutrients to cell wall materials of fruits.[267] Potassium is present in apple juice at *ca.* 1 g l^{-1}, sodium, calcium, and magnesium in the range 16–131 mg^{-1}, iron at 1.5–4.7 mg l^{-1}, and manganese, copper, and zinc at 0.22–0.41 mg l^{-1}.[43]

Antioxidants and Oxygen

Presence of oxygen in fruit juices can cause adverse effects. Oxidized juices lose their freshness and acquire a dull, tired taste. Citrus oils are very susceptible to oxidation to terpines, d-limonene having a harsh turpentine taste.[119] Oxidation also causes color changes, with browning of apple juices being a recognized problem.

In soft drinks and fruit juices, oxygen levels are likely to be low, minimized through the use of hot filling, pasteurization, and antioxidants.[119] If products are in nonoxygen-permeable packaging and filled to minimize headspace, oxygen levels are also likely to be below that needed for mold growth. PET plastic bottles allow slow oxygen permeation,[219] dissolved levels *ca.* 1–3 ppm are common, and require addition of antioxidants for prolonged shelf life. Antioxidants react preferentially with the available oxygen, thereby reducing product oxidation until the antioxidant is consumed. Natural antioxidants are present in many fruits, eg, tocopherols present in citrus peels.[288] Organic acids also act as antioxidants or in synergy with antioxidants.[106] Ascorbic acid (vitamin C) is an excellent antioxidant and is present in orange juice at between 260 and 840 ppm.[116] The German RSK (Richwerte und Schwankungsbreiten bestimmter Kennzahlen) guidelines stipulate a minimum of 200 ppm in single-strength orange juice.[103] Other juices such as apple, contain less than 100 ppm although 318 ppm has been reported.[148] The high susceptibility of apple juices to rapid oxidative browning has led to the practice of adding ascorbic acid (500 ppm) to fruit during milling or to the expressed apple juice.[148]

Sulfites, while recognized as preservatives, have a long history as antioxidants, maintaining fresh aromas in juices.[162] In the presence of metal ions, sulfites are preferentially oxidized to sulfates. Other antioxidants, BHA (butylated hydroxyanisole), or BHT (butylated hydroxytoluene) are used to protect oil-based flavors before addition to beverages.[279] These may be present in soft drinks, diluted with flavors, to a final concentration around 1 ppm.

Preservatives

Preservatives permitted in soft drinks include sorbic acid and sorbate salts, benzoic acid and benzoate salts, SO_2, sulfite, bisulfite, and metabisulfite salts, and esters of p-hydroxybenzoic acid, and parabens.[50] The antimicrobial activity of preservatives in drinks is discussed in Section 31.5.2.

31.3 THE MICROBIOLOGY OF THE MANUFACTURING PROCESS

31.3.1 The Manufacturing Process

Fruit juice manufacture typically occurs close to the areas of fruit production, to minimize transport costs and deterioration of fruit. After delivery, fruit is processed to remove leaves, stems, and other foreign items; washed; milled; and crushed (Figure 31-3).[165] Certain fruits may require destoning. The juice is usually extracted by pressure and the solids removed in "juice polishing" operations that may involve clarification, decanting, centrifugation, or filtration. Pectolytic enzymes may be added to the fruit mash[79,120] to improve extraction and aid clarification. Single-strength juice is bulky, requiring large storage volume and incurring high transportation costs. Juices are therefore commonly concentrated at the site of production. Juice is heated to just below 100°C, vacuum is applied, and excess water removed by evaporation.[212,214] Volatile components, such as aroma oils, are recovered and added back to concentrates after cooling. Depending on how the final concentrate will be handled, one of three options can be pursued: (1) Add preservatives (eg, benzoic acid, sulfite, or combinations) and fill into drums for ambient storage; (2) fill into drums and freeze (frozen concentrated orange juice exported from Brazil accounts for the majority of juice imported into the US[133,188]); and (3) aseptically fill into sterile drums for ambient storage.

Soft drinks manufacture is essentially a simple operation of dissolving flavors, acid, and sugar in water and dispensing into appropriate containers (Figure 31-4). Sugar either comes as granules and is dissolved to make a 67° Brix syrup or is ready-supplied in the syrup form. During sugar dissolving, temperatures of typically 85°C/30 seconds are employed. Separately, other components of the product are combined (ie, fruit juice, flavors, acid, and antioxidants). This is mixed with simple sugar syrup to form the final syrup. This syrup (approximately 40–50° Brix) is proportioned with water to produce a final product of approximately 7–10° Brix. This is filled into the relevant consumer packaging, eg, can, glass, or PET bottle, sealed, and entered into the distribution system.

Microbial stability may be achieved in a number of ways. Products may be sterilized in-pack using a

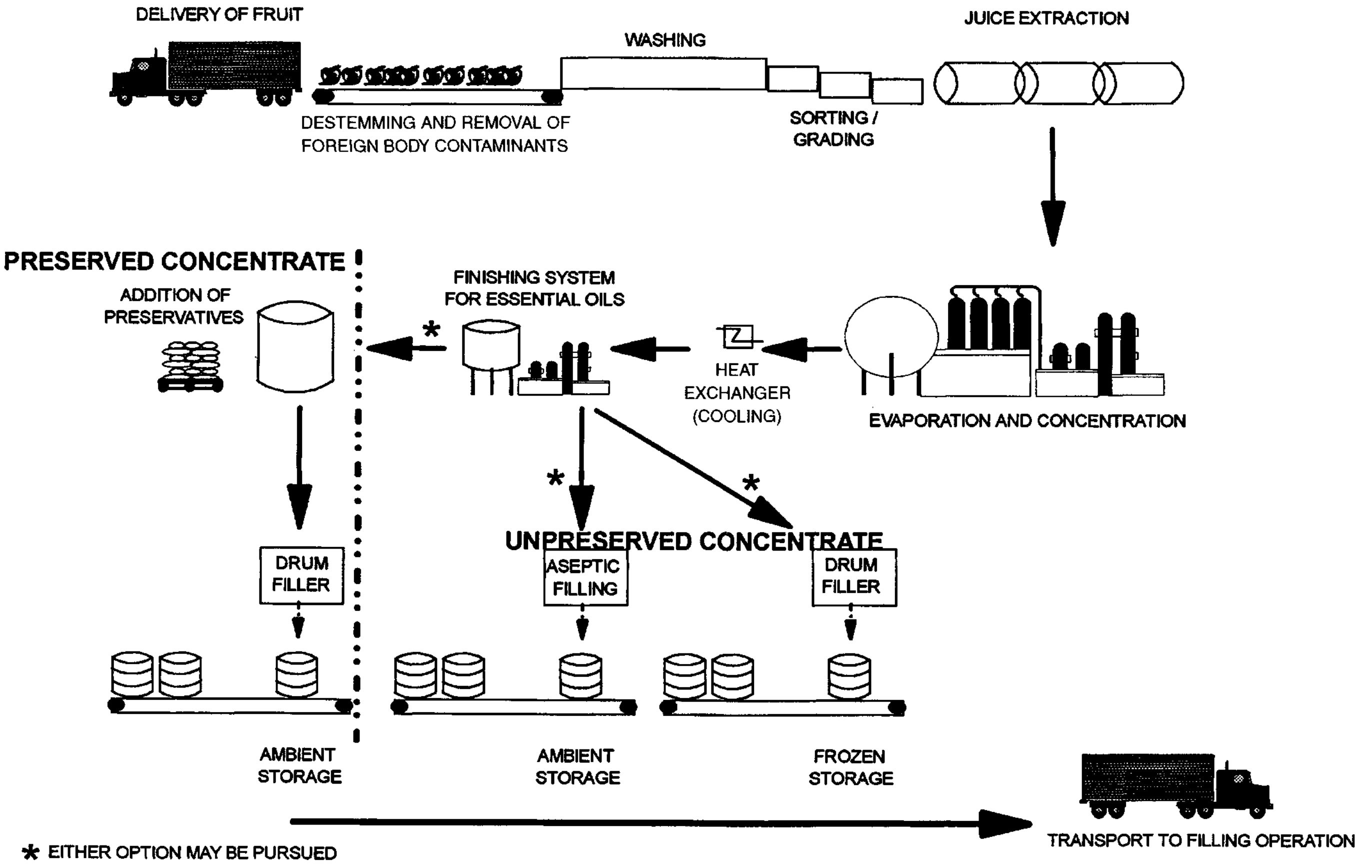


Figure 31-3 Schematic Diagram of a Fruit Juice Processing Plant.

Tunnel pasteurizer, sterilized in-line using a Plate pasteurizer and aseptically filled, in-line pasteurized and hot-filled, preservatives may be added, or the product given a flash, in-line pasteurization to lower the microbial load, in addition to use of preservatives. Products, such as fresh-pressed fruit juices may be preserved by chill storage for a limited time.

31.3.2 The Microbiology of Production

Sources of Infection

Fruit and fruit juices are major sources of yeast and mold contamination. The surfaces of fresh fruit are often contaminated with large numbers of molds,[194] particularly where the surface has been broken. Mold ascospores are widely distributed in soil; and fruits coming into contact with the ground are particularly prone to contamination with ascospores.[183] Fruit, at all stages of development, represents a major natural habitat for many species of yeast.[78] Shriveled mummified fruits, in particular, are excellent environments for osmophilic yeasts, many of which can spoil fruit juices.[27,68,286,287] Consequently, fruit juices contain large numbers, >100, of yeast species derived from the orchard/vineyard.[23,27,58,144,153,179,194,215,283] Other yeasts contaminate juices from the juice-extracting equipment.[27] Most yeasts will not proliferate in juices and can be regarded as hygiene indicators. In addition, yeasts are generally heat sensitive and few will survive concentration of juice or pasteurization. Yeast ascospores can survive temperatures up to 10°C higher than vegetative cells.[69]

Sugar is handled in a number of forms; granulated bagged, granulated stored in silos, or dissolved in water to give 67° Brix syrup, this latter form is known as simple syrup. Granulated sugar has a very low moisture content and as such will not support growth of microorganisms. Microbiological growth in sugar syrup will be slow because of the high osmotic potential produced. Yeasts reported in sugars and syrups include *Candida apicola, C. lactis condensi, Citeromyces matritensis, Cryptococcus albidus, Pichia anomala, P. membranaefaciens, P. guillermondii, Saccharomyces cerevisiae, Schizo-*

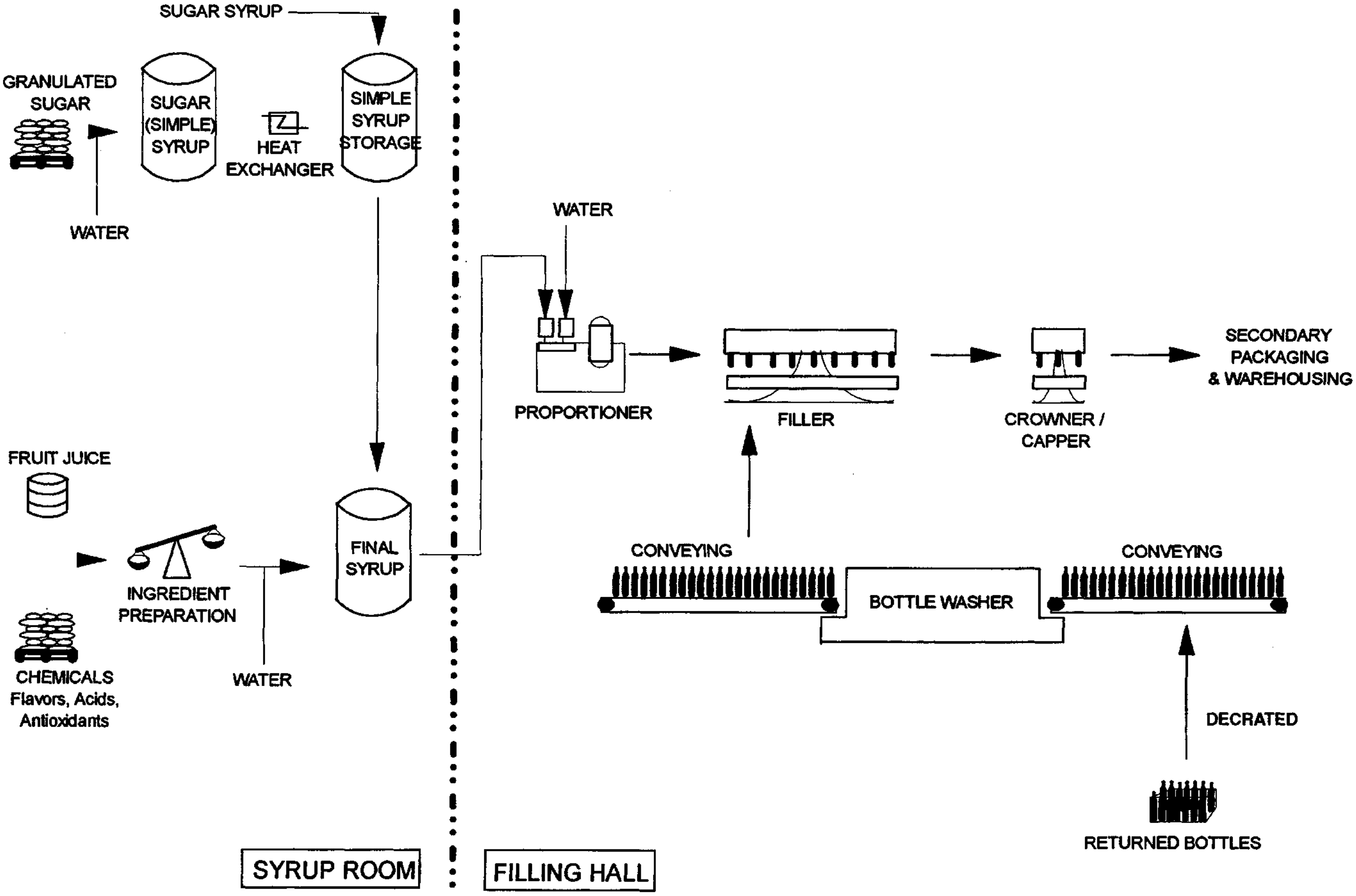


Figure 31-4 Schematic Diagram of a Soft Drinks Processing Plant.

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saccharomyces pombe, Torulaspora delbrueckii, Zygosaccharomyces bailii, Z. rouxii, and *Z. florentinus.*[34,245–247,285,299] While contamination levels in sugars are typically fairly low, if there are deficiencies in storage conditions so that either granules become damp or syrups diluted, there will be an increased risk of microbial spoilage. Good Manufacturing Practice (GMP) should be employed to control these hazards, eg, storage of granulated sugar in dry areas. When handling concentrated syrup, formation of yeast and mold ascospores is a potential area for concern, particularly where products are later heat processed; typical heat treatments are insufficient to destroy ascospores.

Water is the principal ingredient in soft drinks and the quality of water should be closely monitored. Water may be treated by a number of processes, for example, ion exchange or reverse osmosis. Also included within the water treatment processes will be one, or a number of decontamination steps, typically using chlorine or UV light treatment. Water treatment steps must be carefully and routinely maintained in order to avoid high levels of microorganisms.[229] Sand and carbon filters should be backwashed and disinfected.

Additives such as acidulants, flavorings, colorings, antioxidants, or preservatives are also possible sources of contamination. Preservatives in particular are often assumed to be sterile but this is not so. Preservatives are usually added as sodium or potassium salts with little antimicrobial action until placed in a low pH beverage. Spilled preservatives can also allow adaptation to preservative by spoilage yeasts.

Packaging can be a source of microbial contamination where bottles are washed and reused, particularly in tropical environments.[45] Empty bottles coated with sugary residues are frequently permitted to incubate at ambient temperature for prolonged periods. Return of bottles to the factory represents a source of microbes, adapted to growth in preserved beverages. Ideally, returned bottles are kept segregated from production areas. Even after stringent bottle washing, problems can occur, notably mold pellicles remaining in bottles and mold ascospores in bottles, surviving pasteurization.

Aerial contamination in the environment of a soft drinks plant can affect contamination levels both in terms of geographic location[227,237] and local factors. Nearby sources of microbes, such as breweries, wineries, storage/processing of fruit or vegetables, or even sources of dust will obviously have an adverse effect. Such contamination is often highly seasonal, late summer presenting the greatest risk, because of fruit and vegetable harvesting, ambient warmth and dryness, all increasing the dust level that then enters via open windows and doors in the plant. Incidents are known where *Debaryomyces hansenii* and *Candida lambica (Pichia fermentans)* infections occurred in late summer, following demolition of nearby buildings and dust ingress through open windows. Aerial contamination obviously depends on the microbial loading and composition of the air. Mold spores usually predominate. About 95% of aerial flora over fruit orchards were found to be molds.[3] Many molds have evolved mechanisms for violent spore ejection into the air, ballistospores, or projection of ascospores by bursting asci.[128] Analysis of summer air showed the mold flora to contain very high proportions, 60% to 70%, of ascospores and ballistospores. Ingold[128] concludes "mold spores are essentially airborne" indicating that the aerial contamination route is likely to be most significant for molds.

Insects are probably the most important aerial vector for yeasts.[27] The relationship between yeasts and insects is only just becoming recognized,[145] despite the report a century ago that yeast will multiply in the crops of insects if fed on sugars.[109] Yeasts generally serve as food in one or more of the developmental stages of insects. Fruit flies (*Drosophila*) are often attracted to soft drinks, fruit juices, and fermented beverages. *Drosophila* in their natural environment consume many yeasts and exhibit a preference for certain species.[61] Studies of *Drosophila* spp. from oak-pine forests isolated 56 yeast species, many of which are significant to soft drinks spoilage (Exhibit 31-1). Yeast communities of insects were influenced significantly by insect habitat[146] but given the diversity of yeasts carried, insects such as *Drosophila* have potential to be very significant vectors of yeasts in soft drinks production.

Exhibit 31-1 Yeasts Isolated from *Drosophila* spp. Relevant to the Microbiology of Soft Drinks and Fruit Juices (bold type indicates current taxonomy)

Candida guilliermondii = ***Pichia guilliermondii***
Candida krusei = ***Issatchenkia orientalis***
Candida parapsilosis
Debaryomyces hansenii
Kloeckera apiculata = ***Hanseniaspora urarum***
Kluyveromyces thermotolerans
Pichia membranaefaciens
Saccharomyces cerevisiae
Torulaspora delbrueckii
Zygosaccharomyces fermentati
Zygosaccharomyces florentinus

Source: Data from M.A. Lachance, D.G. Gilbert, and W.T. Starner, Yeast Communities Associated with *Drosophila* Species and Related Flies in an Eastern Oak-Pine Forest: A Comparison with Western Communities, *Journal of Industrial Microbiology,* Vol. 14, pp. 484–493, © 1995; and J.A. Barnett, R.W. Payne, and D. Yarrow, *Yeasts: Characteristics and Identification,* 2nd ed., © 1990, Cambridge University Press.

Fruit Juice Manufacture

Fresh fruit can be regarded as routinely heavily contaminated with microorganisms, the majority being yeasts. Fruit juices from healthy fruit may contain 10^3–10^5 CFU ml^{-1}, whereas those from damaged fruit can be several orders of magnitude higher.[299] Small soft fruit with a large surface area, such as grapes, may yield as many as 10^7 yeasts g^{-1}.[269] An unpreserved fruit juice containing 10^3 fermentative yeasts ml^{-1} can be expected to spoil within 2 days at ambient temperature, although if refrigerated a shelf life of several weeks is possible.[268] However, most juices are given a heat treatment soon after extraction, either as pasteurization or in concentrate manufacture (Figure 31-3). During concentration, juices are subject to temperatures of 99°C for about 12 seconds,[46] a treatment sufficient to kill most vegetative microbes.[94] Fruit juice concentrates still contain some surviving microbes unable to grow at low pH and low water activity,[46] but are more likely to be recontaminated after cooling. While low a_w and pH in concentrates restricts spoilage to osmotolerant yeasts,[242,286,287] other yeasts may be found, often inhabiting a watery condensation slick, floating on top of the concentrate. Osmotolerant yeasts often grow slowly, particularly in sugar syrups but also in dilute media. Concentrates are an environment where osmotolerant yeasts can compete successfully against faster growing, osmotically sensitive yeasts.[66] Freezing or addition of preservatives to concentrates prevents microbial growth until reconstitution to single-strength juice. 70° Brix apple concentrate is stable for up to three years if chilled.[28]

Soft Drinks Manufacture

Soft drinks manufacture, including the bottling of fruit juices, is essentially a very rapid operation allowing little time for microbial growth in the bulk of the liquids. Final syrups for dispensing are prepared batchwise (Figure 31-4), sufficient for a few hours bottling. Production lines routinely handle 36,000 liters h^{-1} into glass or 30,000 liters h^{-1} into PET bottles.[45] Within soft drinks operations, microbial infection can occur through the raw materials, particularly juices, rarely through aerial vectors, or through microbial growth within the plant. Fruit juices are typically received in a concentrated, preserved format. While the microbiological quality of these juices is an important area to control, correct handling is also of key importance. Contamination and growth of preservative-resistant osmophiles, such as *Zygosaccharomyces rouxii* or *Z. bailii* in concentrates, can lead to contamination of the soft drinks operation by preadapted preservative-resistant spoilage yeasts. However, it has been estimated that 95% of yeast infections of soft drinks are caused by poor plant hygiene.[90] Sugary residues from soft drinks can build up on equipment used throughout the production process.[230,239,240,241] Yeasts and molds are capable of growth on these residues and can subsequently contaminate products. In order to avoid excessive contamination from equipment, all equipment should be hygienically designed without crevices or "dead spaces" to prevent accumulation and stagnation of product. Equipment should also be easily cleanable. Regular cleaning is essential to avoid build-up of microorganisms. Items of equipment most commonly associated with microbiological contamination and to which particular attention should be given are proportioners/proportioning pumps; carbonators; fillers, particularly filling heads; and sealing equipment/cappers (Figure 31-4). Yeasts found on equipment are often not spoilage organisms. Sand et al.[241] described *Candida sake* as the species most commonly found on proportioning pumps, yet to the authors knowledge never having caused spoilage. *Pichia anomala (Hansenula anomala)* is also commonly found and can cause spoilage.

Preservation procedures applied to soft drinks are not sufficient to guarantee complete sterility within packs, either by pasteurization or by using preservatives. Yeasts have been reported in 40% of commercial fruit juices[80] while Obeta and Ugquanyi[186] reported that up to 27% of mango and tomato juices contained heat-resistant molds. Contaminants are usually low in number and the great majority of such contaminants are not a cause for concern, being unable to grow or cause product spoilage. However, presence of fermentative spoilage yeasts, such as *Z. bailii*, at any level in a beverage is unacceptable. *Z. bailii* can grow and cause spoilage from one cell/package.[71,204] In an unpreserved beverage at ambient temperature, *Z. bailii* can cause spoilage within days, although it may take several weeks to develop sufficient pressure to rupture metal cans. Preservatives at normal concentrations will slow the growth of *Z. bailii* but spoilage will eventually occur, usually after between 4 and 8 weeks. The closed shelf life for pasteurized or preserved soft drinks is usually 6 to 12 months. It is often not appreciated that shelf life in this instance is not terminated by slow microbial spoilage but by organoleptic deterioration, often due to depletion of antioxidants and product oxidation.

A common sales outlet for soft drinks is through bars and restaurants; soft drinks are often prepared on-site via a "post-mix" concentrate and dispensing system. Post-mix systems frequently involve concentrated soft drink to which 4 to 5 parts water is added. Concentrates are supplied in stainless steel kegs or bag-in-box systems. These are linked via a dispensing system, where the product is proportioned with still or carbonated water. Microbiological spoilage has been reported in these post-mix systems. Problems have been attributed to incorrect handling of kegs; bad design of kegs, such that they cannot be correctly cleaned; nonadherence to daily hygiene routines around the dispensing head; and poor design of proportioning systems resulting in back flow of diluted syrup and subsequent spoilage. The organisms responsible for spoilage are similar to those commonly associated with soft drinks; however, there have been more frequent incidents involving Gram-negative bacteria, eg, *Gluconobacter*.[235] Spoilage is controlled with high levels of weak-acid preservatives and adherence to a regular cleaning and disinfection program. There is an increasing trend away from kegs toward bag-in-box because of the cleaning issue noted above.

31.4 MICROBIAL SPOILAGE OF FRUIT JUICES AND SOFT DRINKS

31.4.1 Characteristics of Microbial Spoilage

Soft drinks and fruit juices are acidic products, usually containing substantial amounts of fermentable sugars. Such an environment is ideal for spoilage by yeasts, molds, and for those few acid-tolerant bacteria. Spoilage in this context means microbial growth and metabolism, as opposed to incidental presence of nongrowing organisms. It is therefore not surprising that spoilage of soft drinks and fruit juices predominantly involves yeasts and molds. Out of 27 product recalls in 1993 in the US, 9 involved spoilage by yeasts and molds; other recalls were not due to microbial spoilage.[12] Microbial spoilage of soft drinks can cause clouds, sediments, and particulates. Fungal growth results in submerged mycelial masses or surface pellicles or destruction of cloud in citrus juices.[310] Taints may be produced, such as musty flavors formed from phenols by actively growing molds. Most spoilage yeasts are highly fermentative, forming ethanol and CO_2 from sugars. Ethanol imparts a characteristic sweet taste but excess pressure because of gas production is potentially much more serious. Yeast fermentations have been known to bulge cartons, split cans, and cause explosions in glass or plastic bottles or even aluminum kegs. An exploding bottle not only represents direct hazard but usually splashes the packaging of nearby containers. Mold growth on splashes may render large consignments unsalable.

The microbial ecology of most environments is likely to involve large numbers of growing organisms, smaller numbers of microbes unable to grow but held in stasis by factor(s) in the environment, and a few dying contaminants. Spoilage of soft drinks/fruit juices involves metabolism by growing microbes. Other microbes present as nongrowing contaminants represent a measure of hygiene in production.[69,71] Presence of such organisms should not be viewed with complacency because alterations in production can allow adventitious growth. For example, the presence of the ubiquitous yeast *Debaryomyces hansenii (Candida famata)* normally indicates poor hygiene, but can easily become a spoilage problem, given sufficient oxygen.

In a sweeping microbiological generalization, soft drinks and fruit juices are acidic broth media, usually high in sugars but low in assimilable nitrogen, phosphates, and oxygen. Low pH strongly suppresses bacteria and low temperature or high C/N ratio also favors fungi rather than bacteria.[283,287] Lack of oxygen, while restricting growth of many yeasts, almost entirely suppresses growth of filamentous molds. This results in a microbial flora dominated by fermentative yeasts, with molds and acid-tolerant bacteria where oxygen is sufficient.

31.4.2 Yeasts

Yeasts are fungi that exist predominantly as single cells. Some 600 species are currently recognized.[23] Many imperfect yeasts, classified as Deuteromycetes, are nearly identical to spore-forming perfect forms (Ascomycetes or Basidiomycetes), eg, *Candida famata* is the imperfect form of *Debaryomyces hansenii,* and similarly, *Brettanomyces* and *Dekkera.* Presence of either form indicates an environment suitable for both.

Major Spoilage Yeasts of Soft Drinks, Fruit Juices, and Concentrates

In 1985, Pitt and Hocking[204] assembled a list of 10 yeasts predominantly responsible for food spoilage, where foods had been processed according to normal standards of good manufacturing practice. Of these, seven are noted spoilers of soft drinks and fruit products: *Brettanomyces intermedius, Saccharomyces bailii, S. bisporus, S. cerevisiae, S. rouxii, Schizosaccharomyces pombe,* and *Torulopsis holmii.* The remainder can be regarded as opportunistic spoilers: *Candida krusei, Debaryomyces hansenii, Kloeckera apiculata,* and *Pichia membranaefaciens.* Of the yeasts reported in soft drinks, fruit juices, and concentrates, we have assembled a list of the 11 most significant spoilage organisms (Exhibit 31-2), in terms both of frequency of occurrence and significance of damage. In addition to those on the Pitt and Hocking list, we have included *Dekkera bruxellensis, Saccharomyces bayanus, Torulaspora delbrueckii,* and *Zygosaccharomyces microellipsodes. Dekkera naardenensis (Brettanomyces naardenensis)* and *Dekkera bruxellensis (Brettanomyces intermedius)* have been implicated in spoilage of many foods, including beer and soft drinks. Most *Brettanomyces* species have similar spoilage properties[204]: sediments, floating particles and, characteristically, acetic and sour-fruity off-flavors. These yeasts are predominantly spoilers of low-nutrient soft drinks, such as cola, tonic, soda water, or clear lemon.[90,257] Fermentation is slow and spoilage may not be apparent for weeks. *Dekkera naardenensis* is unable to ferment sucrose while *Dekkera bruxellensis* ferments glucose and sucrose. These yeasts are characteristically sensitive to preservatives. *Saccharomyces cerevisiae* is the yeast commonly associated with brewing and baking. This ubiquitous yeast is an entirely domesticated organism with no known natural habitat.[224] Its appearance as a spoilage organism of soft drinks often indicates yeasts escaped from wineries, breweries, or bakeries. Brewer's yeast is the main contaminant in installations producing both beer and fruit juices.[168] Absence of *S. cerevisiae* in soft drinks plants in Iraq may be significant[237] given the lack of alcoholic bev-

Exhibit 31-2 Most Significant Soft Drinks Spoilage Yeasts, in Terms of Both Frequency of Occurrence and Magnitude of Damage (bold type indicates current taxonomy)

Low nutrient soft drinks/colas:
- *Brettanomyces intermedius* = ***Dekkera bruxellensis***
- *Brettanomyces naardenensis* = ***Dekkera naardenensis***
- *Saccharomyces carlsbergensis* = ***Saccharomyces cerevisiae***

Fruit juice–containing soft drinks:
- *Saccharomyces uvarum* = *S. heterogenicus* = ***Saccharomyces bayanus***
- *Saccharomyces carlsbergensis* = ***Saccharomyces cerevisiae***
- *Candida holmii* = *Torulopsis holmii* = ***Saccharomyces exiguus***
- ***Schizosaccharomyces pombe***
- *Saccharomyces bailii* = ***Zygosaccharomyces bailii***
- *Saccharomyces bisporus* = ***Zygosaccharomyces bisporus***
- *Saccharomyces microellipsodes* = ***Zygosaccharomyces microellipsodes***
- *Saccharomyces rouxii* = ***Zygosaccharomyces rouxii***

Fruit juice concentrates:
- ***Saccharomyces cerevisiae***
- ***Schizosaccharomyces pombe***
- *Saccharomyces rosei* = *S. delbrueckii* = ***Torulaspora delbrueckii***
- *Saccharomyces bailii* = ***Zygosaccharomyces bailii***
- *Saccharomyces bisporus* = ***Zygosaccharomyces bisporus***
- *Saccharomyces rouxii* = ***Zygosaccharomyces rouxii***

Source: Data from J.A. Barnett, R.W. Payne, and D. Yarrow, *Yeasts: Characteristics and Identification,* 2nd ed., © 1990, Cambridge University Press.

erage production in this area. There are many strains and variants of *S. cerevisiae*; 130 are listed by Barnett et al.[23] Strains show considerable variation in resistance to preservatives[302,304] from moderate to highly resistant. *S. cerevisiae* is a highly fermentative yeast, fermenting glucose, fructose, or sucrose with ease. Growth of this yeast is fast; optimal doubling time for diploid/polyploid strains is 2 hours while haploid strains can double in 75 minutes. Spoilage results in alcoholic fermentation, pressure build-up, and clouds and haze in the beverage.

Saccharomyces bayanus is currently recognized as a distinct species, but with considerable homology with *S. cerevisiae, S. paradoxus,* and *S. pastorianus.*[296,297] *S. bayanus,* previously known as *S. uvarum,* can be regarded as near-identical with *S. cerevisiae* in terms of spoilage of soft drinks.

Saccharomyces exiguus (Candida holmii, Torulopsis holmii) is encountered in spoilage in brines and shows vigorous fermentation of a limited range of sugars, including glucose and sucrose. It is moderately preservative resistant and growth is poor above 35°C. Appearance and spoilage characteristics are generally similar to *S. cerevisiae.*

Schizosaccharomyces pombe is a fission yeast, in appearance long cylinders with rounded ends, growing by dividing centrally. This is a highly fermentative yeast, fermenting both glucose and sucrose. It is osmotolerant, growing in 50% glucose, highly preservative resistant, and grows well at 37°C. Fortunately, this a relatively uncommon spoilage yeast, perhaps in consequence of its very slow growth rate, doubling time *ca.* 4 hours.

Torulaspora delbrueckii (Saccharomyces rosei) is a round budding yeast often found on fruit such as grapes. It is osmotolerant, growing in 60% glucose, and is noted for spoilage of unpreserved concentrates.[242] It is a fermentative yeast, fermenting glucose easily but sucrose poorly. *T. delbrueckii* is frequently found as a contaminant of soft drink plants but rarely caused soft drinks spoilage because of its sensitivity to preservatives, such as benzoic acid.

Zygosaccharomyces bailii is a yeast renowned both for its phenomenal resistance to preservatives and for the damage caused in spoilage of soft drinks, juices, concentrates, jams, wines, and ciders. Spoilage is characterized by sour taste, sediment, and slow fermentation up to very high pressures. *Z. bailii* grows relatively slowly, with a doubling time of *ca.* 2.5 hours. It has been described as a fructophile, fermenting fructose strongly in preference to glucose, and sucrose very poorly. This phenomenon is because of selection by the sugar transport permeases of *Z. bailii.*[86,266] The natural habitat of *Z. bailii* appears to be fruits, fermenting fruits, or mummified fruits[68,70] where its osmotolerance may be an advantage. It grows well in concentrates, at up to 50% glucose. *Z. bailii* has further advantages as a spoilage yeast in that it can grow from extremely low inocula, as few as 1 cell l^{-1}.[71,204] Cells can also become adapted to preservatives. Prior exposure to spilt preservatives in the factory can result in *Z. bailii* able to survive preservative concentrations 4 to 5 times the normally permitted levels. Interestingly, while *Z.*

bailii is a noted spoilage organism of wine, it can also make a positive contribution to flavors in wines during fermentation.[221]

Zygosaccharomyces bisporus is a yeast very closely related to *Z. bailii* and sharing its spoilage characteristics of slow fermentation, excess pressure, sediment, and haze. It is also a fructophile, and slightly more xerophilic than *Z. bailii*, growing at 60% glucose.[286] Variations between strains means considerable overlap, but generally *Z. bisporus* is less preservative resistant than *Z. bailii.*

Zygosaccharomyces microellipsodes has been described as "less dangerous than *Saccharomyces cerevisiae*"[236] in spoiling soft drinks but is capable of rapid spoilage of carbonated soft drinks, fermenting sucrose and glucose, and causing pressure increase and particulates. It is widely distributed in soft drinks plants and is moderately resistant to preservatives. It is unusual in its growth temperature maximum of just over 30°C.

Zygosaccharomyces rouxii is the most significant spoilage organism in concentrates, preserved or otherwise, in consequence of its extreme osmotolerance.[189] Its water activity limits are 0.62 a_w in fructose[250] and 0.65 a_w in glucose/glycerol.[286] It has been reported in a wide variety of foods and is also a significant cause of spoilage in soft drinks and fruit juices. Spoilage results from fermentation of sugars, particularly fructose. This yeast cannot ferment sucrose but, like *Z. bailii*, is reported capable of growth from very low contamination levels.[204] *Z. rouxii* also has a high degree of resistance to preservatives.

Recent research on spoilage yeasts involving physiology and RNA sequencing has uncovered a new species, *Zygosaccharomyces lentus*. This organism, isolated from spoiled orange juice and drinks, wine, and tomato ketchup, is fermentative, shows preservative resistance comparable with *Z. bailii* and tolerance to 60% sugar. Unusually, this yeast grows at 4°C and could pose problems in juices stored at chill temperature (H. Steels & S.A. James, pers. comm.—British Yeast Meeting, 1998).

The "Forensic Approach" to Soft Drinks Microbiology

Another approach to the microbiology of soft drinks is the "forensic approach" to contamination and spoilage.[71] This relies less on names of organisms and more on the ecological characteristics of groups of organisms. From this, likely significance of microbes in the factory can be determined and, in reverse, the microbes can be used to reveal the history of a beverage and likely sources of contamination. Contaminants are divided into four groups: Group 1—Spoilage; Group 2—Spoilage and Hygiene; Group 3—Hygiene; and Group 4—Aliens. Table 31-2 lists those yeasts reported in soft drinks and indicates their likely role in this context. Table 31-3 similarly lists yeasts reported in concentrates. Group 1 characteristics in soft drinks are fermentation of sugars, generating gas, and preservative resistance, examples being *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae.* Group 2 includes *Debaryomyces hansenii,* whose presence normally indicates poor hygiene but can cause spoilage where conditions allow, eg, by oxygen ingress. Acetic acid bacteria are also included in this group of microbes. Group 3—Hygiene indicators, includes the ubiquitous yeast *Rhodotorula glutinis.* Group 4—Aliens includes microbes that are out of place in this environment. It is possible that some aliens reported in the literature may represent misidentification. Interestingly, recent taxonomic studies have shown a close relationship between Group 1 soft drinks spoilage yeasts.

The Zygosaccharomyces/Torulaspora *Superfamily*

Assessment of the dominant spoilage microorganisms of soft drinks, juices, and concentrates reveals a number of characteristics, common to all or present in many. These are: (1) fermentation of sugars, (2) fructophilly, preference for fructose, (3) preservative resistance, and (4) osmotolerance.

Recent taxonomic studies[132] based on 18s ribosomal sequences have revealed a close relationship between many of the dominant spoilage organisms of soft drinks (Figure 31-5). *Zygosaccharomyces bailii* and *Z. bisporus* are very closely related, and linked to *Z. rouxii* and *Z. mellis.* Also close are *Torulaspora delbrueckii* and *Z. microellipsodes.* This independent study confirms the physiological similarity of these spoilage yeasts and suggests that these are taxonomically genuine species.

31.4.3 Molds

The fungal kingdom in general is much better adapted than bacteria to life at low pH. Low pH is preferred by fungi; indeed for most fungi, a pH value near neutrality represents an upper limit to growth. Taxonomically, the fungi are grouped largely according to production of sexual spores. This is widely recognized as an artificial distinction; many deuteromycetes are near-identical to other fungi in all aspects other than sexual spores. As a result,

Table 31-2 Yeasts Reported in Soft Drinks, Fruit Drinks, and Colas and Their Significance in This Environment

Brettanomyces anomalus = ***Dekkera anomala***	S	*Kloeckera apiculata* = ***Hanseniaspora uvarum***	S
Brettanomyces clausenii = ***Dekkera anomala***	S	*Kluyveromyces cicerisporus* = ***Kluyveromyces marxianus***	S
Brettanomyces intermedius = *Dekkera intermedia* = ***Dekkera bruxellensis***	S	*Kluyveromyces veronae* = ***Kluyveromyces thermotolerans***	S
Brettanomyces naardenensis = ***Dekkera naardenensis***	S	***Lodderomyces elongisporus***	S
Candida boidinii	H	***Pichia etchellsii***	H
Candida curiosa = ***Cryptococcus curiosus***	H	***Pichia fermentans***	H
Candida guillermondii = ***Pichia guilliermondii***	H	*Saccharomyces bailii* = ***Zygosaccharomyces bailii***	S̲
Candida intermedia	H	*Saccharomyces bisporus* = ***Zygosaccharomyces bisporus***	S̲
Candida krusei = ***Issatchenkia orientalis***	H	*Saccharomyces chevalieri* = *S. italicus* = *Candida robusta* = ***Saccharomyces cerevisiae***	S̲
Candida lusitaniae = ***Clavispora lusitaniae***	H	***Saccharomyces exiguus*** = *Candida holmii* = *Torulopsis holmii*	S̲
Candida parapsilosis	S/H	*Saccharomyces florentinus* = ***Zygosaccharomyces florentinus***	S
Candida reukaufii = ***Metschnikowia reukaufii***	H	***Saccharomyces kluyveri***	S
Candida sake = *Eutorulopsis sake*	H	*Saccharomyces microellipsodes* = ***Zygosaccharomyces microellipsodes***	S
Candida solani	H	*Saccharomyces montanus* = ***Zygosaccharomyces fermentati***	S
Candida tropicalis	H	*Saccharomyces rosei* = *S. delbrueckii* = *S. vafer* = ***Torulaspora delbrueckii***	S
Candida vartiovaarai = ***Torulopsis vartiovaarai***	H	*Torulopsis candida* = ***Candida saitoana***	H
Candida willianus = *Saccharomyces willianus* = *S. uvarum* = ***Saccharomyces bayanus***	S	*Torulopsis glabrata* = ***Candida glabrata***	H
Debaryomyces hansenii = *Candida famata* = *Torulopsis famata*	S/H	*Torulopsis stellata* = ***Candida stellata***	H
Debaryomyces phaffii = ***Debaryomyces polymorphus***	H	*Trichosporon fermentans* = ***Geotrichum fermentans***	H
Hanseniaspora melligeri = ***Hanseniaspora guilliermondii***	H		
Hansenula anomala = *Candida pelliculosa* = ***Pichia anomala***	S/H		
Hansenula californica = ***Williopsis californica***	H		

S indicates Spoilage-Group1, S̲ being noted spoilage yeasts; S/H indicates Spoilage/Hygene-Group 2; H indicates Hygene-Group 3 (R.R. Davenport - personal communication;[71]). Bold lettering indicates current taxonomy.[23] *Source:* Data from[18,58,90,210,215,227,233,236,237,238,241,264,292,293,312]

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many fungi are described as having two forms, perfect and imperfect, and taxonomically belong to different genera, eg, *Byssoclamys* is the ascospore-producing perfect form of *Paecilomyces* (Table 31-4).

Given sufficient oxygen, many molds thrive in soft drinks and fruit juices, typically growing near the surface as mycelial mats, sometimes forming aerial mycelia and sporulating. Juices may be clarified by release of extracellular pectolytic enzymes.[310] Because fruit juices are usually pasteurized, surviving organisms are predominantly heat-resistant molds or heat-resistant ascospores that may be activated by heat[88]; yeasts are generally heat sensitive.[21,75] Heat-resistant molds causing spoilage of soft drinks and fruit products include *Aspergillus ochraceus, A. fischeri, A. tamarii,* and *A. flavus, Byssoclamys nivea, B. fulva, Paecilomyces variotii, Neosartorya fischeri, Eupenicillium brefeldianum, Phialophora mustea, Talaromyces flavus, T. trachyspermus,* and *Thermoascus aurantiacum.*[21,88,291] Spoilage is also commonly caused by *Penicillium notatum, P. roquefortii,* and *Cladosporium* spp., molds also noted for survival of ozonation, filteration, and UV treatment.[104] Heat-resistant molds and ascospores are sensitive to preservatives[183] but are best controlled by low oxygen, careful selection of raw materials, and good plant hygiene.

31.4.4 Bacteria

The majority of bacteria will not grow in acidic media. Many are rapidly killed in such an environment, but a few are able to thrive at low pH, namely acetic acid bacteria, *Acetobacter* and *Gluconobacter* spp., lactic acid bacteria, *Lactobacillus* and *Leuconostoc* spp., Lancefield Group N streptococci,

Table 31-3 Yeasts Occurring in Fruit Juice Concentrates and Their Significance in This Environment

Candida glabrata = *Torulopsis glabrata*	*H*
Candida guilliermondii = ***Pichia guilliermondii***	*H*
Candida krusei = ***Issatchenkia orientalis***	*H*
Candida lambica = ***Pichia fermentans***	*H*
Candida lusitaniae = ***Clavispora lusitaniae***	*H*
Candida magnoliae = *Torulopsis magnoliae*	*H*
Candida maltosa	*H*
Candida parapsilosis	*H*
Candida sake = *Eutorulopsis sake*	*H*
Candida stellata	*H*
Candida tropicalis	*H*
Cryptococcus humicolus	*H*
Cryptococcus laurentii	*H*
Debaryomyces hansenii = *Candida famata* = *Torulopsis famata*	*H*
Hanseniaspora guilliermondii	*H*
Hanseniaspora occidentalis	*H*
Hansenula anomala = ***Pichia anomala***	*H*
Hansenula saturnus = ***Williopsis saturnus***	*H*
Kloeckera apiculata = ***Hanseniaspora uvarum***	*H*
Kloeckera corticis = ***Hanseniaspora osmophila***	*H*
Kluyveromyces veronae = ***Kluyveromyces thermotolerans***	*H*
Lodderomyces elongisporus	*H*
Oosporidium margaritiferum	*H*
Pichia membranaefaciens = *Pichia sylvestris* = *Candida mycoderma* = *C. valida*	*H*
Rhodotorula mucilaginosa	*H*
Saccharomyces chevalieri = ***Saccharomyces cerevisiae***	S
Saccharomyces rosei = *S. delbrueckii* = ***Torulaspora delbrueckii***	S
Saccharomyces uvarum = *S. heterogenicus* = ***Saccharomyces bayanus***	S
Schizosaccharomyces pombe	S
Sporidiobolus salmonicolor	*H*
Torulopsis apicola = ***Candida apicola***	*H*
Torulopsis versitalis = ***Candida versatilis***	*H*
Zygosaccharomyces bailii	S
Zygosaccharomyces bisporus	S
Zygosaccharomyces rouxii	S

S indicates Spoilage-Group1, S being noted spoilage yeasts; S/H indicates Spoilage/Hygene-Group 2; H indicates Hygene-Group 3 [R.R. Davenport - personal communication;[71]]. Bold type indicates current taxonomy.[23] *Source:* Data from [76,90,153,205,231,242,278,282,286,292]

Clostridium butyricum and *C. pasteurianum*, *Alicyclobacillus* (ex-*Bacillus*) *acidoterrestris*, *B. coagulans*, *B. macerans*, and *B. polymyxa*.[46,47,177] Bacterial spores, such as those of *Clostridium botulinum* will not germinate at a pH less than 4.5; although slow growth of *C. botulinum* at pH 4.1 has been reported in laboratory media.[261] *C. pasteurianum* and *C. buytricum* can grow at pH 3.6 and have been reported to spoil fruit juices.[47] *Clostridium* spp. from animal slurry were the causative agent in explosions in pear juice, pH 4.72.[28] Spores of *Bacillus licheniformis* and *B. subtilis* have been shown to germinate in tomato juice at pH 4.4 and to grow, gradually raising the pH to 5.0.[220] Such spores easily survive pasteurization but growth is dependent on availability of oxygen.[219,220]

Bacterial spoilage at low pH is most frequently associated with Gram-negative *Gluconobacter* (*Acetomomas*). These also depend absolutely on the presence of free oxygen for growth and are restricted by gas-impermeable packaging and minimal headspace.[234] Still drinks in plastic beakers are particularly prone to *Acetomomas* spoilage.[229] *Acetomonas* can be detected in large quantities in processing plants[228] but should be contained by normal plant hygiene. It is resistant to normal concentrations of preservatives and dimethyldicarbonate (DMDC), but grows only slowly in fruit juices.[46]

Lactobacilli and *Leuconostoc* spp., the lactic acid bacteria, are known to cause spoilage in fruit juices causing loss of astringency, slime, or ropiness in texture, pressure, turbidity, or buttermilk off-flavors.[21,47,195,226,229,231] Spoilage by lactic acid bacteria can occur where selected and adapted strains are allowed to build up in the plant.[229] Such bacteria can grow in products at pH 2.8 but are relatively heat sensitive[21,231] and lose viability in chilled juices.[195] Fruit juices, particularly tomato, may also be spoiled by species of *Bacillus*, *B. coagulans*, *B. macerans*, and *B. polymyxa*. The characteristic "flat-sour" spoilage of tomato juice is caused by *B. coagulans*, a thermophilic bacterium growing from 30°C to 60°C.[96,263] More recently, another acidophilic, heat-resistant spore-forming organism, *Alicyclobacillus* (formerly *Bacillus*) *acidoterrestris*, has caused concern. This can survive pasteurization, grows well at low pH, and is capable of spoiling fruit juices, fruit juice blends, and lemonade, producing off-flavors and visible growth.[48,77,164,200,270,311]

31.5 PRESERVATION AND PRESERVATION PROCEDURES

The multiple factors involved in preventing microbial spoilage of soft drinks and fruit juices including intrinsic factors present as part of the beverage, and factors and procedures instigated deliberately for suppression of microorganisms, can be broadly divided into physical and chemical methods of preservation.

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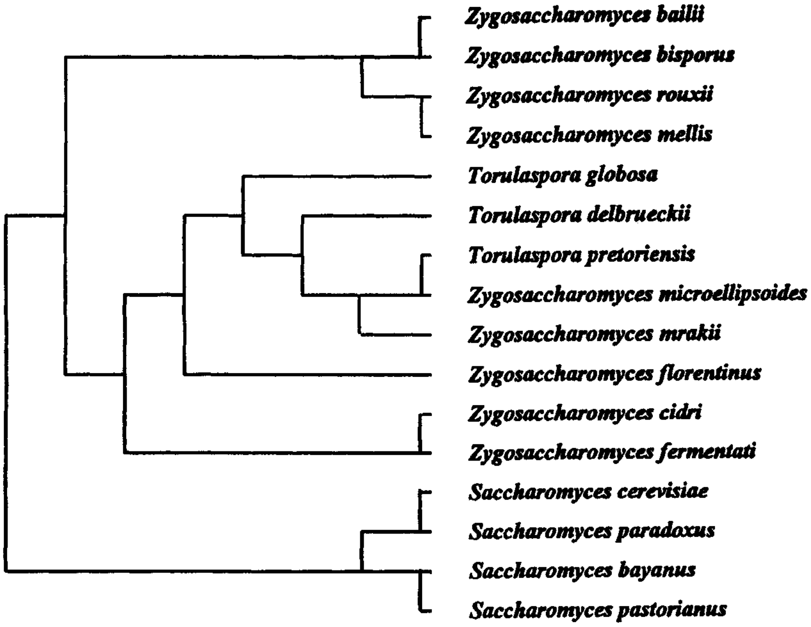

Figure 31-5 Interrelationships between *Zygosaccharomyces*, *Torulaspora*, and *Saccharomyces* Species of Soft Drinks Spoilage Yeasts. *Source:* Reprinted with permission from S.A. James, M.D. Collins, and I.N. Roberts, Use of an RNA Internal Transcribed Spacer Region to Distinguish Phylogenetically Closely Related Species of the Genera *Zygosaccharomyces* and *Torulaspora*, *International Journal of Systematic Bacteriology*, Vol. 46, pp. 189–194, © 1996, Society of General Microbiology.

31.5.1 Preservation—Physical

pH

pH value is probably the single most important factor in preservation of soft drinks and fruit juices. pH impinges on many aspects of preservation, in addition to direct interactions of protons with microbes. The impact of acidulants in taste and antimicrobial action is pH affected. Low pH greatly increases the effect of pasteurization, synergistically increases the effects of weak-acid preservatives, and also acts as an additional hurdle to the growth of microbes.[150]

The pH of most soft drinks and fruit juices is below 4.0. The great majority of heterotrophic bacteria are unable to grow at such a low pH value[35] with certain notable exceptions.[237] This is in marked contrast to the fungal kingdom, which is well represented in those few organisms able to grow at pH 0.0.[252] The optimum pH growth range of most fungi lies between 3.0 and 6.5. Spoilage yeasts and molds in rich media can grow at very low pH values (Figure 31-6). The yeast *Saccharomyces cerevisiae* struggles at pH 2.2, whereas the mold *Byssoclamys fulva* grows at pH 1.6. Most spoilage yeasts and molds grow at pH values greater than 2.0. These values are of minimum pH for initiation of growth. Growing yeasts and molds acidify media (Figure 31-7)[149,258] and may be found growing at a pH lower than those shown here. Pitt and Hocking[201,204] quote a minimum pH for growing *Saccharomyces cerevisiae* of 1.5 and *Candida krusei* of pH 1.3. Surprisingly, the preservative-resistant yeast *Zygosaccharomyces bailii* is not exceptionally resistant to low pH.[204] Proton efflux from yeast cells may be a consequence of raising internal pH but also may serve as a mecha-

Table 31-4 Relationships between Perfect (Sexual Spore-Forming) and Imperfect (Nonsexual Spore-forming) Mold Genera, Most Commonly Associated With Spoilage of Soft Drinks and Fruit Juices

Perfect Genera—Ascomycetes	*Imperfect Genera—Deuteromycetes*
Byssoclamys	*Paecilomyces*
Neosartorya	*Aspergillus*
Taleromyces, *Eupenicillium*	*Penicillium*
	Cladosporium
	Phialophora

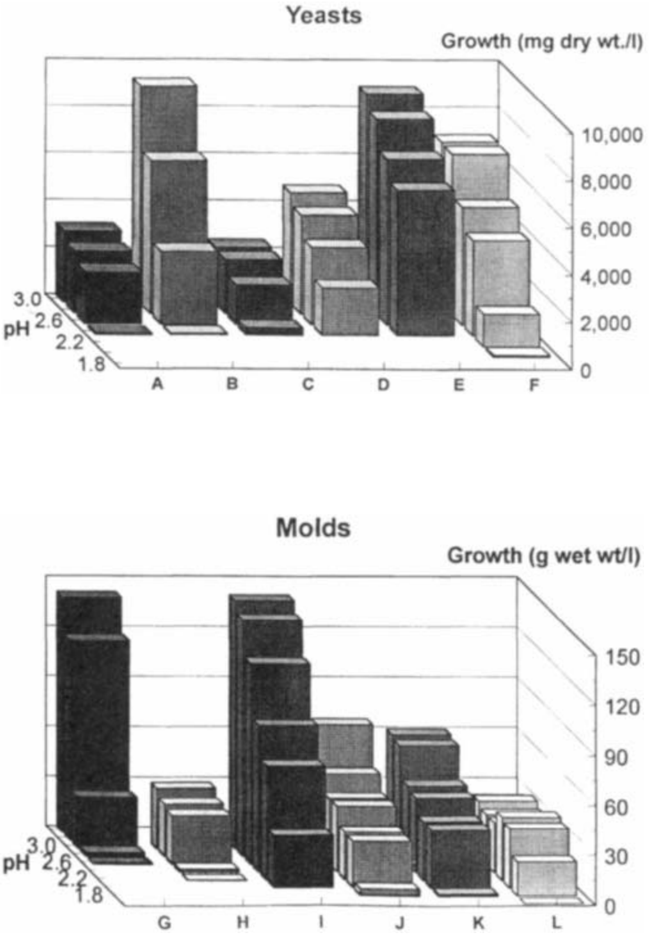

Figure 31-6 Low pH Limitation of Growth of Soft Drinks Spoilage Yeasts and Molds. Organisms were cultured in rich media, yeasts-YEPD; molds, Potato/Dextrose at 25°C for 1 week in aerobic shake flasks. Yeasts were: A, *Zygosaccharomyces rouxii*; B, *Candida parapsilosis*; C, *Schizosaccharomyces pombe*; D, *Zygosaccharomyces bailii*; E, *Debaryomyces hansenii*; F, *Saccharomyces cerevisiae*. Molds were: G, *Aspergillus ochraceous*; H, *Penicillium expansum*; I, *Aspergillus flavus*; J, *Neosartorya fischeri*; K, *Paecilomyces variotii*; L, *Byssoclamys fulva*.

nism to suppress bacterial competition for the available nutrients.

The mechanism of inhibition of yeasts by low pH remains a matter for speculation.[223] Possible sites of action by pH are external and membrane proteins,[62] particularly transport permeases, membranes[31] and acidification of the cytoplasm following penetration of protons into cells. Cytoplasmic acidification may prevent growth by inhibition of pH-sensitive enzymes like phosphofructokinase,[143] by interference in the role of pH_i in cellular signal transduction,[281] or by exhausting cellular reserves of ATP in pumping protons from the cell.[121,251] Most organisms endeavor to keep the cytoplasmic pH close to neutral[222] at least during growth. Stationary phase yeasts are known to allow internal pH to fall to below pH 5.2[53,126,127] in acidic media. Higher internal pH is thought to enable enzymes to function at or near their pH optima and also provide the driving force for active transport.[36] Proton removal from the cytoplasm appears to be a key factor allowing growth at low pH, involving oxidative metabolism,[243] polyol metabolism,[134] and plasma-membrane proton pumps.[110,244,309]

To conclude, low pH is the predominating factor affecting microbial spoilage of soft drinks, largely eliminating bacteria as spoilage organisms. Suppression of fungi by low pH requires a pH lower than 1.5. This is not organoleptically acceptable and other factors must be used in combination with low pH to prevent microbial spoilage.

Water Activity

Water activity (a_w) is a measure of water availability based on the ratio of water vapor pressure of the sample to that of pure water, at the same tempera-

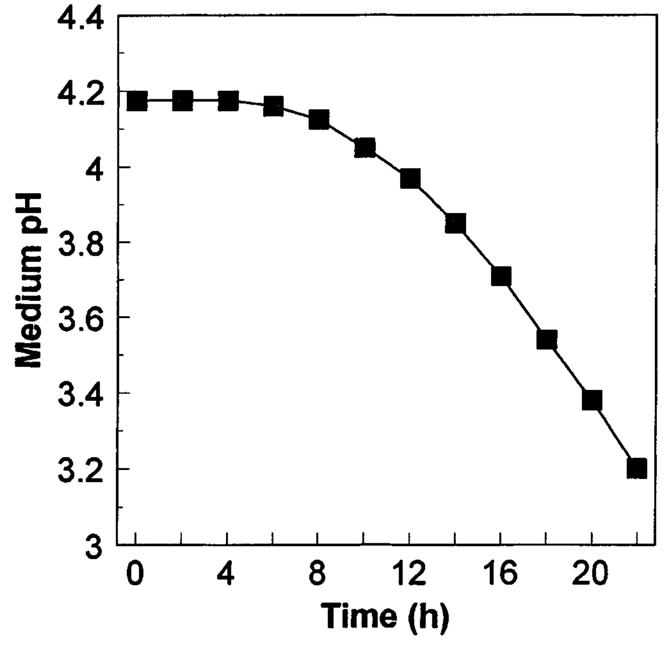

Figure 31-7 Reduction in Medium pH Caused by Yeast Inoculated into Sugar-Containing Media. *Saccharomyces cerevisiae* NCYC 1224 was inoculated, 1 mg dry wt. l^{-1}, into yeast nitrogen base media (Difco), containing 20 g l^{-1} glucose and incubated at 25°C.

ture. The principal solutes in soft drinks are sugars and organic acids. A 10° Brix soft drink has an a_w close to 0.993, and a 65° Brix sucrose concentrate has an a_w of 0.865.[51] Osmophilic or xerotolerant yeasts and molds[286] are defined as those able to grow at water activities less than 0.865[247] or 0.85.[201] Many molds can grow at an a_w of 0.80[51] including the spoilage molds *Aspergillus flavus*, 0.78, and *A. ochraceous*, 0.77.[204] Many yeasts can grow at a_w of 0.90. The average soft drink or fruit juice at 10° Brix has an a_w *ca.* 0.99 and offers little impediment to microbial spoilage.

Many spoilage yeasts of soft drinks are osmotolerant, notably *Zygosaccharomyces rouxii*, 0.62/0.65,[250,286] *Zygosaccharomyces bailii*, 0.80,[204] and *Debaryomyces hansenii*, 0.65/0.75,[65] which also can grow in the presence of 24% sodium chloride.[204] Osmotolerance undoubtedly enables growth of these organisms in fruit juice concentrates, which can act as inocula for spoilage of diluted products. We speculate that osmotolerance may aid infection of soft drinks via survival in concentrates, in mummified fruit, or in gummy droplets of dried soft drinks in unhygienic factories.

Pressure and Carbonation

It is generally recognized that modified atmospheres, particularly CO_2 enriched, can affect the growth of microorganisms (see Chapter 10).[82,136] Sparkling soft drinks contain carbon dioxide under sufficient pressure to have a major impact on microbial growth. Growth of some bacteria can be inhibited by as little as 5% CO_2,[54] and germination of some types of spores prevented.[87] Yeasts and molds are very resistant to such inhibition, 100% CO_2 at atmospheric pressure having little effect. Yeast in every brewery grows and ferments in a saturated CO_2 solution.

Pressurized CO_2 (carbonation) is generally known to be antimicrobial[173,254,260]; an effect that is often confused with a pressure effect or lack of oxygen. At 20°C, 3 volumes carbonation exerts a pressure of 2.6 bar (atmospheres), and 5 volumes, 4.8 bar.[172] Yeasts are inhibited by pressurized CO_2, not by pressurized nitrogen, showing that pressure *per se* has no effect.[154] Similarly, carbonation does not remove all oxygen from solution, indeed pressure will act to increase oxygen concentration. The degree of carbonation required to inhibit yeasts is of the order of 2 bar[2,91] although some 30 bar is required to kill them.[255] Inhibition was greatly increased by low temperature, an effect only partially explained by increased CO_2 solubility.[87] CO_2 in solution is in equilibrium with carbonic acid, bicarbonates, and carbonates and may be regarded as a weak acid with a pK_a of 6.35.[35] CO_2 can cross microbial membranes with great speed[283] and is known to have a central role in pH_i homeostasis. However, the physiology of growth inhibition argues against a role for CO_2 as a weak-acid preservative, although carbonation may exacerbate inhibition by weak acids.[152] Carbonation may interfere with amino acid uptake in yeast[142] but less so in bacteria.[82] Yeast cell division is prevented but cell volume continues to increase and DNA synthesis is unimpaired.[154] Work by Eyton-Jones[91] suggested inhibition of cell wall synthesis, possibly mediated via membrane-bound enzymes.

Pasteurization

Pasteurization is probably the most effective single method of preservation of soft drinks and beverages. The most probable target of heat damage is DNA, although membranes, RNA, ribosomes, and enzymes are also likely targets.[111] Pasteurization can be achieved "in pack" through use of a Tunnel pasteurizer, or by in-line pasteurization and hot-filling bottles, which are then inverted to heat the caps. Inline pasteurization and aseptic filling is a developing option. The advantages of pasteurization are low costs, reliability, and no carryover into the product. Disadvantages are capital investment, physical and organoleptic damage to the product, heat resistance of mold ascospores, and limited heat stability of plastic bottles.

Vegetative yeast and mold cells and asexual spores (conidia) are relatively heat sensitive. Two minutes at 52°C will kill most brewing yeasts,[155] while 10 minutes at 60°C kills almost all vegetative mold cells.[75,202,203] D_{55} for vegetative cells of *S. cerevisiae* is 0.90 minutes.[268] Effectiveness of pasteurization is altered by presence of sugars, salts, lipids, proteins, or preservatives[32,185]; sucrose in particular decreases its effectiveness,[63,64,108] and low pH increases the kill. Examples of pasteurization for orange juice range from 10 to 15 minutes at 70° to 75°C.[21] Carbonated products may also be pasteurized effectively, albeit at a slightly lower temperature.

Ascospores of molds present the greatest challenge to pasteurization of fruit juices. Ascospore-forming yeasts were shown to be marginally more heat resistant than non–spore-forming strains.[69,210] Mold ascospores, particularly those of *Byssoclamys fulva*, are considerably more heat resistant, with D values up to 12 minutes at 90°C.[25,33,141,203,209] Bacterial spores, such as those of *Bacillus licheniformis* and *B. subtilis*, can survive pasteurization in tomato juice. D_{90} values quoted are 29.9 minutes and 29.5 minutes, respectively.[219] Growth of *B. subtilis,* and to a lesser extent of *B. licheniformis,* is reported to be restricted by oxygen-impermeable packaging.[220]

Chilled Storage

Soft drinks and fruit juices may be preserved for a limited length of time by chilled storage. This is often used to extend the open shelf life of fruit juices, where it is recommended that after opening, products are chilled and consumed within 3 days (Figure 31-1). Most spoilage yeasts and molds are able to grow at 5°C, although slowly and temperatures close to zero are required to halt the growth of most spoilage yeasts. Bacteria such as *Lactobacillus plantarum* gradually lose viability in chilled juices.[195] Some fungal growth and spoilage is possible down to –7°C.[135,171,193] Molds, such as *Penicillium italicum,* can grow in chilled juices at 0°C; use of oxygen-impermeable packaging can double the shelf life of juices from 35 to 65 days.[310]

31.5.2 Preservation—Chemical

All microbes require certain key nutrients in order to survive or grow. In general, soft drinks contain fewer and more dilute nutrients than fruit juices, and consequently are much more microbiologically stable. A synthetic lemonade containing only citric acid, sugar, and carbonated water, for example, requires much less preservation than a fruit juice. Determination of limiting nutrients in soft drinks can be a difficult task, unless all other nutrients are present to great excess. Several nutrients can be at low levels, each becoming limiting if the concentration of others is raised, a situation identical to that recently highlighted in bacterial growth in drinking water.[169]

Sugars

With the exception of zero-calorie soft drinks, fruit juices and soft drinks contain excess sugars as carbon sources and are relatively low in other nutrients. The types of sugar present can have a profound effect on spoilage. Preservative-resistant yeasts, *Zygosaccharomyces bailii, Z. rouxii,* and *Z. bisporus,* utilize sucrose poorly if at all,[204] while the presence of fructose in a beverage positively encourages these organisms. Fructophily is closely associated with osmophilic spoilage yeasts,[287] an effect mediated by preferential uptake.[86,266]

Acidulants

Gardner[106] described the functions of acidulants as (1) flavoring agents, (2) buffers controlling pH, (3) preservatives, and (4) synergists to antioxidants. As antimicrobial agents, the effects of acidulants might be ranked in decreasing order of importance: (1) inhibition as a consequence of lower pH, (2) action as poor weak-acid preservatives, (3) inhibition by chelation of metal salts, and (4) modification of preservative action via buffering.

Inhibition of microbes by low pH (high proton concentration) is discussed in Section 31.5.1. Acidulants may act to exacerbate the effect of low pH by acting as proton carriers across the plasma membrane, in effect acting as weak-acid preservatives. Such a mechanism of action was suggested by observations that yeasts could tolerate lower pH if a strong acid like HCl was used as an acidulant, rather than phosphoric, citric, malic, tartaric, or particularly acetic acids.[201] These acids are known to have an antifungal effect at high concentration, 1% to 5%,[131,138,160,218] and also to inhibit bacteria at low pH.[137] Relative lack of preservative activity by di- and tri-basic acids, succinic and citric, may be because of the inability of these acids to diffuse through the plasma membrane lipids.[201] These acids have extremely low partition coefficients.[151] This is supported by reports of slow transport of citric and succinic acids into yeast.[275]

Many organic acids and acidulants bind metal ions with high affinity, varying widely with acid and ion[157,158]; highly charged ions of transition metals such as Fe^{3+} are bound most strongly. Stability constants for citric acid metal complexes are several orders of magnitude greater than those of other acidulants.[157,158] Inhibition of *Clostridium botulinum* by citric acid was found to be relieved by Ca^{2+}, Mg^{2+}, Fe^{2+}, or Mn^{2+} ions.[113] Therefore, it is probable that inhibition by citric acid is partly mediated by metal chelation, at least at higher pH where the chelating citrate ion predominates.

High concentration of acidulants in soft drinks has a considerable impact on buffering capacity (Figure 31-2). This may affect the impact of weak-acid preservatives on spoilage microbes. Weak-acid preservatives are more effective at low pH. Yeasts introduced into a glucose-containing medium immediately acidify the broth (Figure 31-7), enhancing the effect of weak-acid preservatives. Bacteria such as *B. subtilis* have been shown to raise the pH of tomato juice.[220] If acidulant buffers are present, the pH will be much less susceptible to change.

Microbial Nutrients: Minerals and Vitamins

Microbial stability of beverages is known to depend on the nutrient content, particularly nitrogen, phosphorus, sulfur, and vitamins.[217] Magnesium limitation slows growth of yeasts,[39] nitrogen or phosphate limitation causes cessation of growth and fermentation,[272,300] whereas lack of vitamins eg, inositol, can lead to cell death.[118,294] While the majority of molds are able to utilize nitrate as a nitrogen source, yeasts are generally more fastidious, requiring ammonium ions or amino acids. These are present in fruit juices, although nitrogen availability limits yeast growth in apple and grape fermentation.[125,174,272] Optimum level for growth varies between microbes, but generally concentrations of 1.75–5 mM NH_4 appear ample for bacteria, yeasts, and molds.[39] Pitt and Hocking[204] quote an example of an Australian product containing 25% fruit juice, withdrawn because of microbiological spoilage, and replaced with a stable beverage containing synthetic flavors and color. The critical nutrient in this instance is nitrogen.

Phosphates are also present in relatively low concentration in most soft drinks and fruit juices. However, phosphate requirements by microorganisms are much lower than those of nitrogen source—*ca.* 50 μM phosphate.[39] This can limit microbial growth in soft drinks.[300]

Micronutrients such as potassium, sodium, calcium, magnesium, manganese, copper, and zinc, while in low concentration in fruit juices, are in excess of requirements for microbial growth to 10^7–10^8 yeast cells ml^{-1}.[9,253] Micronutrients in grape musts are ample for yeast fermentation.[144] Recently, it was shown that removal of iron from grape juices using ion-exchange resins can render them less prone to spoilage.[95] Microbial vitamin requirements vary widely even at the species level, but are generally at the trace level. As little as 0.01 ng of vitamin B_{12} can cause a definite growth response.[9] Citrus and other fruit juices contain low concentrations of thiamin, riboflavin, and folates.[116,197] Addition of small quantities of vitamins to apple juices may promote yeast fermentation.[298] Thiamin deficiency can arise during storage of fruit juices, particularly when treated with sulfites.[256]

Oxygen

Of all the antimicrobial factors associated with soft drinks and fruit juices, oxygen availability is likely to have greatest impact on molds. There are very few anaerobic molds, exceptions being obligate anaerobes inhabiting intestines of ruminants[190] or low-oxygen stagnant water.[85] Of molds associated with spoilage, Miller and Golding[170] reported growth inhibition at 0.56 mg l^{-1} oxygen (1% v/v), Follstad[97] reported growth restriction at 0.5%, and spore germination inhibition at 0.1% oxygen.[276] Mold oxygen requirements are strain dependent but generally in excess of 0.05 mg l^{-1}. Fermentative yeasts, such as *Saccharomyces cerevisiae* or *Zygosaccharomyces bailii*, can be regarded as facultative anaerobes. Fermenting cells of *Saccharomyces cerevisiae* utilize oxygen for synthesis of unsaturated fatty acids and sterols, and trace amounts of these in media abolishes any oxygen requirement.[7,8] Lack of oxygen also restricts spoilage of soft drinks by many acid-tolerant bacteria. Spores of *Bacillus licheniformis* and *B. subtilis* are barely affected by pasteurization of fruit juices but, unexpectedly, require oxygen for spore germination and outgrowth in tomato juice at pH 4.4.[219,220] Gluconobacter are similarly oxygen dependent. Use of more gas-permeable plastic packaging may increase spoilage by these organisms.[220]

Natural Inhibitors

Many fruit juices and soft drinks contain natural inhibitors of microbial growth. These include essential oils or phenolic compounds,[74,196,284] polyphenols in apple juices, in particular chlorogenic acid,[148] limonene in citrus juices and peels,[178] herbal oils in colas,[24] phenolics in grapes,[22] or the antibacterial and antiviral components of tea.[181,289] Natural antimicrobial compounds appear not to be present in soft drinks in sufficient concentration to affect the growth of yeasts or molds. Possibly bacteria are more sensitive to these inhibitors. Phenolics in red grapes were reported to inhibit aciduric bacilli.[167,270] Similarly, use of thyme in alcoholic soft drinks[16] may inhibit bacteria through its carvacrol content.[59,60,140] Natural oleoresins have been used to inhibit growth of bacteria in alcoholic beverages for over 7,000 years.[163]

Weak-Acid Preservatives

Legally permitted preservatives of soft drinks and fruit juices are few in number. Those most commonly encountered are examples of weak-acid preservatives, sorbic acid, benzoic acid, and SO_2/sulfites. SO_2 has been documented as an antimicrobial in beverages for over three centuries.[225]

Weak-acid preservatives appear to share a common mode of action, despite their various chemical structures. All become increasingly potent as antimicrobial agents at acidic pH values, more being required in soft drinks at higher pH (Figure 31-8). In aqueous solution, weak acids exist in pH-dependent equilibria between uncharged acid molecules and charged anions. The proportion of undissociated acid increases as the pH declines. It is generally assumed

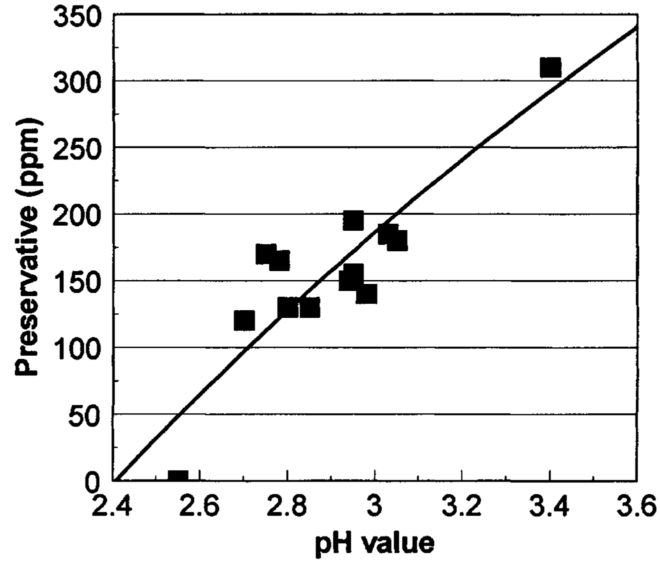

Figure 31-8 Scatter Plot of the pH of Soft Drinks against Preservative Content (Sorbic Acid + Benzoic Acid). Samples of preserved soft drinks were randomly selected from UK supermarkets in 1994.

that only undissociated acids have antimicrobial activity, although some activity by anions has been proposed.[83] The mechanism of action of weak-acid preservatives is thought to involve diffusion of lipophilic acid molecules through the plasma membrane into the cytoplasm[273,305] and dissociation into charged ions. Dissociation releases protons and the cytoplasm becomes increasingly acidic. Acidification of the cytoplasm may prevent growth by inhibition of glycolysis,[143] by prevention of active transport,[100] or by interference with signal transduction. pH_i is increasingly recognized as having a role in signaling.[281]

Resistance and adaptation to weak-acid preservatives has been reported in *Zygosaccharomyces bailii.*[302,303] Resistance may be due to a lower internal pH in resistant yeasts[35] as suggested in *Saccharomycodes ludwigii.*[274] Expression of a sorbate efflux pump has also been suggested[301,304,305] to account for development of resistance, but disputed.[56] The proton pumping H^+-ATPase has also been shown to be stimulated in *S. cerevisiae* in response to weak acids[89,121] although this mechanism appears unlikely in *Zygosaccharomyces bailii* given its poor growth at low pH.

Parabens; Esters of p-hydroxybenzoic Acid

Parabens is the collective term for the esters of para-hydroxybenzoic acid, which are permitted and occasionally used in soft drinks. These comprise the methyl, ethyl, and propyl esters, of which the longer propyl chain confers greatest antimicrobial activity[1] but lowest solubility.[50] In comparison to bacteria, fungi are much more susceptible to parabens.[72]

Parabens can be regarded as weak acids, the parahydroxyl group dissociating at high pH with a pK_a value of 8.5.[83] However, the mode of action of parabens appears distinct from that of weak-acid preservatives.[100] Inhibition is not pH dependent; parabens are efficient inhibitors at pH 7.0 and above (Figure 31-9A). No cross-adaptation to parabens was developed by yeasts grown in weak-acid preservatives[304] and parabens showed synergistic inhibition with several weak-acid preservatives.[216] It is possible that the main action of parabens is against cell membranes.[35] Reinterpreting the data of Oka,[187] Davidson[72] showed that inhibitory concentrations of parabens were directly proportional to that dissolved in the lipid phase. Parabens caused membranes to leak 260 nm–absorbing material[105] and are known to affect active transport in the plasma membrane.[81,100]

Dimethyldicarbonate (DMDC) and diethyldicarbonate (DEDC)

DEDC and DMDC (Velcorin™) can be used as effective sterilants for soft drinks, particularly when the microbial target comprises yeasts.[208] DEDC has been known as a fungicide for 40 years.[117] However, DEDC was banned in the US in 1972 because of erroneous fears of carcinogen formation[191] and DMDC has largely replaced DEDC.

Microbes are rapidly killed by addition of dicarbonates, faster kill being achieved by lower pH (Figure 31-9B), higher concentrations, and temperatures.[67,191,192] Dicarbonates break down rapidly in aqueous environments and thus pose no risk to the consumer, but offer no long-term preservative action. DEDC has been termed a "cold pasteurization"[50] acting only to sterilize but offering no extension of open shelf life.

31.5.3 The Inoculum Effect

The microbial load carried by a beverage will often determine whether a product will suffer spoilage. Large, healthy inocula will obviously shorten the time to spoilage and shelf life of the beverage.[38] The efficiency of many antimicrobial treatments is also dependent on inoculum. Heat treatments designed to cause a 5-log reduction in microbial count are ineffective if 10^8 cells ml^{-1} are present. Preservative efficacy by DMDC,[280] sulfites,[129,183] benzoic

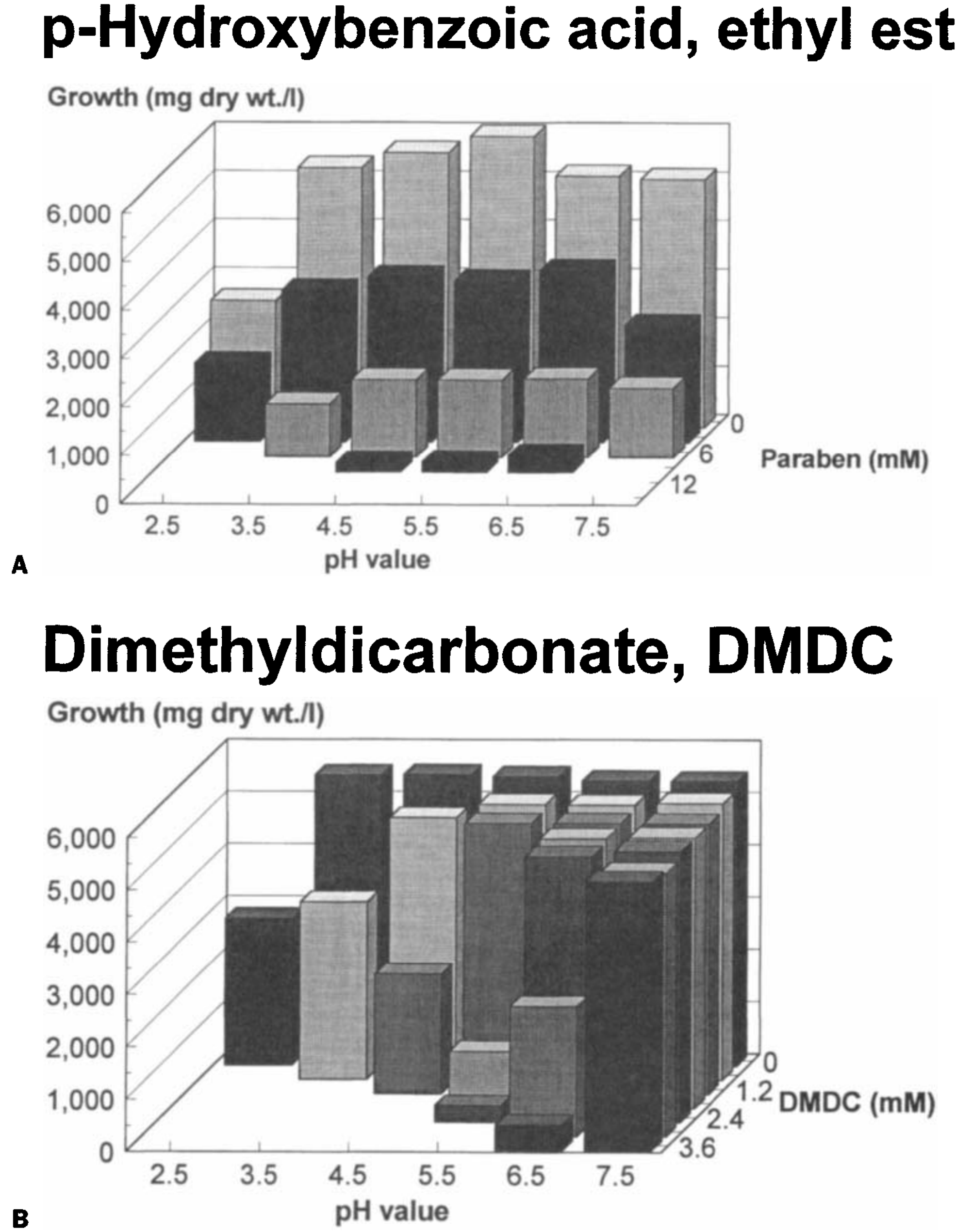

Figure 31-9 The Effect of pH on Inhibition of *Saccharomyces cerevisiae* X2180-1B. **A**, by Parabens, p-Hydroxybenzoic Acid, and Ethyl Ester. **B**, DMDC, Dimethyldicarbonate. Yeast, 1 mg dry wt l^{-1}, was inoculated into matrices of flasks, 50 mls YEPD, at the pH and preservative content indicated. Growth was measured after 3 days shaking at 30°C.

acid,[183,248] and sorbic acid (Figure 31-10) all vary with the inoculum level. More preservative is required to prevent growth from large inocula. Reduction in microbial load to 10 ml^{-1} or fewer will have a considerable impact on product stability. Possible explanations for inoculum effect include adsorption of preservatives by microbial cells, cell walls are known to adsorb organic acids,[147] stochastic increase in probability of inclusion of phenotypically resistant cells or mutants within inocula, or sufficient cell density to maintain cell to cell growth signaling. Recent work has proposed extracellular growth signals and in their absence, death of very dilute yeast populations.[211,213]

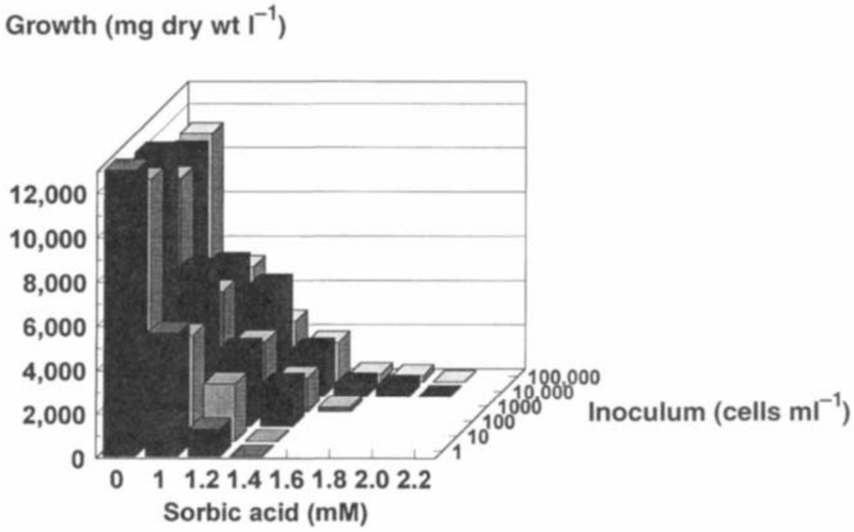

Figure 31-10 The Effect of Inoculum Size on Inhibition of *Saccharomyces cerevisiae* X2180-1B by Sorbic Acid. Yeast was inoculated into YEPD media, pH 4.0, containing sorbic acid at the concentrations indicated. Growth was measured after 9 days on an orbital shaker at 30°C.

31.6 MICROBIOLOGICAL SAFETY: RISKS TO HUMAN HEALTH

The risks to human health from microbial spoilage of soft drinks are relatively minor compared with other foods but nonetheless significant. Greatest risks are probably those associated with injury caused by exploding bottles. Ophthalmologists have noted exploding bottles as a serious cause of eye injury.[114] Spoilage of soft drinks is predominantly by fermentative yeasts, often capable of fermenting to high pressure, 6 bar.[204]

31.6.1 Pathogens

Human consumption of spoilage yeasts is generally unlikely to have adverse effects.[175] Of *ca.* 600 yeast species, very few are pathogenic to healthy humans.[23] However, recent developments in the medical field have resulted in a number of "new and emerging yeast pathogens."[115] Yeasts previously thought innocuous have been shown capable of damaging the human body, often by causing blood infections.[57,115,184] Several of these "new pathogens" can cause spoilage of fruit juices/soft drinks; notably *Candida famata (Debaryomyces hansenii), Candida guillermondii (Pichia guillermondii), Candida krusei (Issatchenkia orientalis), Candida parapsilosis,* and *Saccharomyces cerevisiae.* These new pathogens are almost exclusively of concern in immunocompromised patients and are very unlikely to affect healthy individuals, but add weight to the importance of preventing spoilage of soft drinks. Concern has also been expressed over minor intestinal disorders associated with consumption of *Saccharomyces* and *Zygosaccharomyces* yeasts.[180,290] These probably did not involve any infective process, and may be due to immunological responses to yeast antigens.[156]

Due to the acidity, pathogenic bacteria are rarely found or grow in fruit juices. This has led to the mistaken assumption that acidity causes rapid death of all bacterial pathogens, and stimulated a major food safety initiative by the President of the US.[55] The organism of concern is *Escherichia coli* O157:H7. This strain appears to have acquired a *Shigella*-like toxin gene and is capable of causing illness from a very low infective dose, *ca.* 12 cells.[44,262] Infection can cause death from hemorrhagic colitis and hemolytic uremic syndrome. It has been associated with animal feces from many sources, including cattle, and as a food pathogen in meat, animals, milk, and apple juice.[262] *E. coli* O157:H7 is acid tolerant, showing slight growth in apple juice in large inocula, 10^5 ml^{-1},[313] but from small inocula, 10^2 ml^{-1}, viability was maintained for 12 days and lost gradually over the next 3 weeks. Such behavior stimulated much research into bacterial acid habituation.[26] Several incidents were reported in the US concerning *E. coli* O157 in fresh pressed, unpasteurized apple juices.[42] Thirteen cases of infection were reported in Massachusetts in

1991, 10 in Connecticut in 1996, and 49 in California in 1996, including 1 death.[166] In all these cases, it is suspected that juices were expressed from apples contaminated with fecal material. Coliforms from animal manure have been shown to contaminate apples gathered from the ground in orchards and to persist through juice processing.[112] Resolution of this problem has centered around two approaches. One involves cleaning up in the orchard, removing animals, using fruit only from the tree and cleaning apples before pressing,[166] yet retaining the unpasteurized fresh apple taste in juices, the natural image, and a slight risk. The other approach involves pasteurization or use of preservative. *E. coli* O157 is heat sensitive, D_{52} is 18 minutes.[271] The bacterium loses viability within 2 days if benzoic acid 100 ppm is present.[313] Pasteurization in the presence of benzoic acid is particularly effective, D_{50} -0.64 minutes.[271] Note, in American parlance cider is unpasteurized apple juice, as opposed to the fermented beverage in the UK containing 4% to 9% alcohol.

Other examples of survival of pathogens in fruit juices include *E. coli* O134 contamination of orange juice by road dust[259] and *Salmonella typhimurium* contamination of apple juice, again from animal feces.[10] Research has shown that *Salmonella* spp. can survive and can grow in acidic media, down to pH 4.1[19,52,98,124,261] and raise the pH to tolerable levels.[137] Occurrences of salmonellosis in fruit juices are rare but finite.[10,112] *Salmonella* have been shown to remain viable in apple juice at pH 3.6 for 30 days.[112] The acid-tolerance response of *Salmonella typhimurium* has been shown to provide protection also against organic acids.[19]

Contamination of beverages by viruses, hepatitis A and small round structured viruses (SRSVs), has also been reported. One Australian incident involved 3,000 cases of infection by SRSV in orange juice. The source of contamination appears to have been contamination of potable water by waste water and sewage. *Cryptosporidium,* a parasitic protozoan found in animal feces, is a commonly reported enteric pathogen in humans. Infection usually occurs via contaminated water supplies but *Cryptosporidium* can survive in acidic conditions, pH 2.0 for 1 hour,[40] and has also been implicated in food poisoning following consumption of apple juice[101] and flavored fruit drinks.

31.6.2 Mycotoxins

Mycotoxins are a group of stable, toxic metabolites produced by molds, characteristically in late exponential or stationary phase (see Chapter 53). Mycotoxins are a widely recognized threat to human health[37,102] but expression of mold toxigenic potential is highly variable, even within the species or strain.[139] Given the range of toxigenic fungi in foods, Davis and Diener[73] suggested that "until proved otherwise, moldy foods and beverages should be considered dangerous and not consumed by man or animals." The toxin of particular note in this context is patulin,[13] found in apple juices as a result of mold growth on apples during storage. Patulin is regarded as both carcinogenic and mutagenic in addition to being acutely and chronically toxic,[73] although the degree of patulin toxicity has recently been questioned.[295] Patulin is produced by many species of *Penicillium,* primarily by *Penicillium expansum.* Patulin in apple juices may be eliminated by fermentation to cider or by ascorbic acid addition.[159] A rather arbitrary Maximum Permitted Concentration (MPC) of 50 μg l^{-1} is in force in some countries, such as Norway and Russia[306] and used in other countries as an advisory level.[13] In a survey of 113 Australian apple juices, 65% contained patulin, 29% at concentrations above 50 μg l^{-1}.[306] One roadside sample contained 45mg kg^{-1} patulin.[49]

Other mycotoxins produced by *Penicillium* spp., namely ochratoxin A, citrinin, and penicillic acid, have not been reported in fruit juices. Aflatoxin production is inhibited by caffeine[41,182] but this has been shown not to be true for ochratoxin A, found after mold growth on coffee beans.[15] Caffeine in tea-based soft drinks cannot be assumed to prevent mycotoxin formation. When considering the dangers of mycotoxins in foods, it is important to bear in mind that these are often stable molecules that persist in the food chain. Heat treatments kill molds but can leave high concentrations of mycotoxins to be carried through the manufacturing process into the final product.

31.7 CONCLUDING REMARKS AND FUTURE TRENDS

At present, microbial stability of soft drinks and fruit juices is largely determined by low pH, low oxygen content, pasteurization, and preservatives. Where it does occur, spoilage is predominantly by yeasts but new technologies may change this pattern of microbial spoilage.

31.7.1 New Processing Technologies

Throughout the food and drink industry there is an increasing trend toward natural, healthy products. One of the key criteria used to judge these parameters is the absence of artificial preservatives and additives, these also being controlled by increasingly tighter legislation. Another trend is the move toward plastic packaging. These two trends are providing an increasing number of challenges to the producers of soft drinks and fruit juices in order to consistently manufacture microbiologically stable, acceptable products. In order to address these issues, three main techniques are beginning to emerge. These are (1) hot filling, (2) aseptic filling, and (3) clean/hygienic filling. The first two routes are established technologies and their use is dependent on the willingness of the manufacturer to invest in the technology. However, neither of these methods is suitable for the production of carbonated soft drinks. Consequently, all the major manufacturers within the industry are currently investigating the use of clean, or hygienic, filling.

Hot Filling into Plastic Bottles

This technology is widely used within Japan, and an increasing number of hot-filled products are appearing throughout the world. For this technology, heat-set PET bottles are required, these bottles have a greater thermal stability than standard PET (85°C compared to the 50°C of standard bottles). Other new plastic packaging is being developed, resulting in plastic bottles that are stable to temperatures in excess of 100°C, these bottles include polyethylene-naphthalene (PEN) resin.

Aseptic Filling into Plastic Bottles

Aseptic filling is a technology now well established within the dairy industry. Increasing numbers of soft drinks manufacturers are moving toward aseptic filling into PET bottles. The filling equipment, high-hygiene environment, and associated control systems require a relatively high initial investment. The problems that still have to be overcome in aseptic filling concern the speed of lines, which is slow compared with traditional soft drinks filling operations (200 bottles per minute compared with 500). Furthermore, because of the design of aseptic fillers, it is not possible to fill carbonated products; hence, the development of clean-fill systems.

Clean/Hygienic Filling into Plastic Bottles

A number of different approaches are being taken toward clean/hygienic filling. As outlined in previous sections, the acidic environment of soft drinks limits possible spoilage microorganisms. Consequently, the degree of security designed into aseptic filling systems for low-acid foods is not required. Development of clean-fill systems is being approached from two directions. One route is to modify existing soft drinks production lines in order to reduce microbiological contamination, for example, by enclosing the filler in a clean room environment (class 100 filtered air) and chemically rinsing bottles prior to filling. The second approach is to take standard, slow aseptic filling systems and modify the filling, packaging, and disinfection systems in order to achieve the required increased line speed and ability to fill carbonated products.

Ultra High Pressure

Ultra high pressure (UHP) is currently used commercially within Japan for the production of jam and fruit juices. UHP treatments required are in the region of 4 Kbar. The use of high pressure is primarily seen as a milder option to the use of heat as regards damage to the product. To date, no systems are available for soft drinks, however, it is possible that in the near future, high pressure will be commercially attractive and used in order to vastly improve organoleptic quality.

31.7.2 Improvements in Hygiene: QA and HACCP

Spoilage of soft drinks and fruit juices is predominantly by yeasts. It has been estimated that some 95% of yeast spoilage can be directly attributed to bad factory hygiene.[90] Any improvements in factory hygiene are therefore likely to have a major impact on spoilage, particularly if targeted on likely sources of microbial contamination.

Traditional approaches of microbiological quality assurance (QA) of final products within the soft drinks/fruit juice industry rely on end-product analyses. Methods of sampling and inspection do not deliver reliable control as they are inaccurate, because of the nonuniform distribution of contaminants and results of analyses are retrospective. The ineffectiveness of this approach is illustrated by the number of product recalls as a result of microbiological contamination.[12]

Within the soft drinks industry, as with all other food industries, there is an increasing trend toward the use of quality management systems, such as Hazard Analysis Critical Control Point (HACCP). The HACCP system is a systematic approach to the identification and assessment of hazards and risks associated with the manufacture, distribution, and use of a particular food product,[161] in this case fruit juices and soft drinks. This is led both by increased legislation and recognition of the futility of existing end-product microbiological analysis. For example, recent EC legislation necessitates the identification of hazards using the principles of HACCP for all producers of food and drink.[11] It is hoped that identification of Critical Control Points within the soft drinks industy will lead to more attention to these areas and a consequent decrease in future incidents of spoilage.

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32 Bottled Water

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32.1 INTRODUCTION

Bottled water, as defined by the United States (US) Food and Drug Administration (FDA), is "water that is intended for human consumption and that is sealed in bottles or other containers with no added ingredients except that it may optionally contain safe and suitable antimicrobial agents."[60] In fact, bottled water may include any potable water manufactured, distributed, or offered for sale in any sealed container, including bottles, carboys, cans, jugs, Tetrapaks™, and bags in a variety of sizes from <100 ml to 8 l or more. The source water for bottled water may be springs, wells, municipal systems, or other approved sources. Subsequently, the water may be distilled, carbonated, ozonated, and filtered depending on the quality of source water and the type of bottled water being manufactured; bottled water labeled as "natural" can only have a minimal treatment. Some bottled water may have added flavors, such as fruit juices and simulated flavors (see Exhibit 32-1 for definitions and approved sources and types of bottled water).

The bottled water industry in North America and the United Kingdom (UK) is currently experiencing an annual growth rate of 25%,[30,187] which parallels the growth of this industry worldwide. The dramatic increase in the consumption of bottled water has been prompted by consumers' concern over increasing water pollution, and by objections to offensive tastes or odors from municipal water supplies, and to fluoride, chlorine, and other additives. Increased consumer concern and awareness about health and fitness, changes in social attitudes toward alcohol, along with strong promotional advertising by the bottled water industry has also contributed to the dramatic and rapid growth of this industry over the last few years.[30,177]

The public has an unquestioning impression that bottled water is of good quality because it usually derives from a spring or underground source.[5,66,171] Bottled water is often promoted as pure, impeccably clean, and from scrupulously "protected" sources that are "unharmed by man." There is also the perception that natural-source waters have beneficial medicinal and therapeutic properties.[59,70] Because bottled water is manufactured, distributed, and sold, it is considered a "food" (as are other manufactured beverages) in many countries and is regulated by food standards.

Exhibit 32-1 Definitions of Approved Source Waters and Different Types of Bottled Water

Approved Source[95]	Approved source means the source of water whether it be from a spring, artesian well, drilled well, public or community water system, or any other source that has been inspected and the water analyzed, and found to be of a safe and sanitary quality (ie, is potable) with or without treatment.
Spring and Mineral Water[38,39]	
Spring water	Bottled potable water derived from an approved underground source (bore holes or springs that originate from a geological and physically protected underground water source and not from a public community water supply) that contains less than 500[a] mg l^{-1} total dissolved solids. Spring water may be treated to remove unwanted chemical and microbiological components but may not be labeled as "natural" (see below).
Natural spring water	Same as "spring water," and in Europe must meet the collection requirements of "natural mineral water" (as below) without any treatment to remove bacteriological components.
Mineral water	Bottled potable water obtained from an approved underground source (bore holes or springs that originate from a geological and physically protected underground water source and not from a public community water supply) that contains not less than 500[a] mg l^{-1} of total dissolved solids. In Europe, mineral water may be treated to remove unwanted chemical and microbiological components but may not be labeled as "natural" (see below).
Natural mineral water	**Natural mineral water** is mineral water (as defined above), but must meet the following conditions: it is collected under conditions that guarantee the original bacteriological purity; it is bottled close to the point of emergence of the source with particular hygienic precautions; it is not subjected to any treatments (other than removal of unstable constituents by decantation and/or filtration with the aid of aeration) that modify its essential mineral constituents; and cannot be shipped in bulk. **Naturally carbonated natural mineral water** is a natural mineral water that, after acceptable treatment, replacement of gas and packaging, has the same content of gas as the source. **Noncarbonated natural mineral water** is a natural mineral water that, after acceptable treatment and packaging, does not contain free carbon dioxide in excess of the amount necessary to keep the hydrogen carbonate salts present in the water dissolved. **Decarbonated natural mineral water** is a natural mineral water that, after acceptable treatment and packaging, does not have the same carbon dioxide content as at emergence. A **carbonated natural mineral water** is a natural mineral water that, after acceptable treatment and packaging, has been made effervescent by the addition of carbon dioxide from another origin.
Other Bottled Water[32,95,177]	
Artesian water	Bottled water from a well tapping a confined aquifer in which the water flows freely at the ground surface without pumping. It has been proposed that the collection of the water can be enhanced with the assistance of external pressure so long as such measures do not alter the physical properties, composition, and quality of the water.
Bottled water	Water that is placed in a sealed container or package and is offered for sale for human consumption or other consumer uses.
Carbonated or sparkling water	Bottled water containing carbon dioxide.
Distilled water	Bottled water produced by a process of distillation and has an electrical conductivity of not more than 10 µS cm^{-1} and total dissolved solids of less that 10 mg l^{-1}.

continues

Exhibit 32-1 Continued

Drinking water	Bottled water obtained from an approved source that has undergone special treatment or that has undergone minimum treatment consisting of filtration (activated carbon and (or) particulate) and ozonation or equivalent disinfection process.
Deionized water	Bottled water produced through a deionization process to reduce the total dissolved solids concentration to less than 10 mg l^{-1}.
Fluoridated water	Bottled water containing added fluoride in such an amount that the total concentration of added and naturally occurring fluoride does not exceed 1 mg l^{-1}.
Glacial water	Bottled water from a source that is direct from a glacier. Glacial water shall meet the requirements of natural water.
Natural water	Bottled water (such as spring, mineral, artesian, or well water) obtained from an approved source that is from an underground formation and not derived from a municipal or public water supply system. This water has undergone no treatment other than physical filtration, iron removal, and that has not had any significant change occur in the total concentration of the major ions in comparison with the concentrations occurring in the approved source water.
Purified water	Bottled water produced by distillation, deionization, reverse osmosis, or other suitable process that contains not more than 10 mg l^{-1} of total dissolved solids. Water that meets this definition and is vaporized, then condensed, may be labeled distilled water.
Well water	Bottled water from a hole bored, drilled, or otherwise constructed in the ground, which taps the water of an aquifer. Well water shall meet the requirements of natural water.

[a]The "cut-off" value is 250 mg l^{-1} in the US.[60]

Source: Data from References 32, 38, 39, 60, 95, 177

Like most foods, bottled waters are generally not sterile products. Bottled waters can contain naturally occurring bacteria as well as bacteria introduced during processing or use.

32.2 MICROBIOLOGY OF THE WATER SOURCES

The initial microbiological content of bottled water is dependent on the type of water, the initial source, its location relative to the surface and sources of contamination, the surrounding bedrock and soil constituents, its oxygen and mineral content, as well as the water flow.

Several research groups have shown that many aquifers, down to 4,000 m, contain a variety of microorganisms (Table 32-1), sometimes at concentrations as high as 10^5 to 10^7 cfu g^{-1} of sediment or ml of water.[15,16,68,108,188] Other studies report that the initial indigenous microbial level of spring or mineral water (both the source water and immediately after bottling) may be less than 10^2 cfu ml^{-1}.[18,131,159] No doubt, these variations in bacterial content are related to the type of rock strata, the age of the water, the initial source of water, temperature, oxygen, and nutrient content.[74] Some types of bottled water use potable municipal water as the source; therefore, the initial microbial levels are usually very low. Bacteria indigenous to municipal-treated and other source water may be innocuous or opportunistic (secondary) pathogens (Table 32-1).

32.2.1 Microbiological Contamination of Water Sources

The risk of contamination of bottled water by pathogenic bacteria and parasites is high because both can be found in the source water and can contaminate water during processing. In addition to indigenous bacteria from the natural water source (Exhibit 32-1), bottled water may contain bacteria that enter as contaminants, including a wide range of indigenous saprophytic species (Table 32-1), human pathogenic contaminants,[18] and parasites.[47,151] Fecal contamination presents the most common and widespread source of risk to public health associated

Table 32-1 Bacteria Found at Various Stages in the Production of Bottled Water and Their Medical Significance

Genus	Ground and Spring Water[a]	Municipal Treated Water[b]	A.C./R.O. Filters and Water Softeners[c]	Bottled Water[d]	Gastroenteritis[e]
Achromobacter	+	+	+	+	?
Acinetobacter	+	+	+	+	S
Aerobacter	–	–	+	?	?
Aeromonas	+	+	+	+	P
Alcaligenes	+	+	+	+	S
Bacillus	+	+	+	+	P
Chromobacterium	–	+	+	+	S
Citrobacter	+	+	+	+	S
Clostridium	+	+	+	?	P
Corynebacterium	+	+	+	+	S
Enterobacter	+	+	+	+	S
Escherichia	+	+	+	+	P
Flavobacterium	+	+	+	+	S
Hafnia	–	+	+	+	S
Klebsiella	+	+	+	+	S
Kluyvera	–	–	+	?	S
Legionella	–	+	+	?	S
Micrococcus	+	+	+	+	S
Moraxella	+	+	+	+	S
Mycobacterium	–	+	+	+	S
Proteus	+	+	–	?	P
Pseudomonas	+	+	+	+	P
Salmonella	–	+	–	?	P
Serratia	+	+	+	+	?
Shigella	–	+	–	+	P
Staphylococcus	+	+	+	+	P
Streptococcus	+	+	+	+	P
Yersinia	–	+	–	+	P

(+) - isolated (–) - not isolated; (P) - pathogen causing gastroenteritis
(S) - secondary pathogen-not known if it causes gastroenteritis; (?) - not known

[a] Ground water including aquifers, springs, and underground sources.[16,18,24,46,106,108,135,147,149,168,191]

[b] See references.[35,36,53,67,112,113,144]

[c] A.C. = activated carbon filters, R.O. = reverse-osmosis filters[31,67,111,142,166]

[d] See references.[107,158,161,163,164,177]

[e] See references.[26,64,109,117,167,189]

Source: Adapted with permission from D.W. Warburton, A Review of the Microbiological Quality of Bottled Water Sold in Canada, Part 2, The Need for More Stringent Standards and Regulations, *Canadian Journal of Microbiology*, Vol. 39, pp. 158–168, © 1993, NRC Research Press.

with all types of drinking water. Water (from springs, wells, municipal supplies, and other sources) has been associated with outbreaks or sporadic cases of foodborne illness resulting from enteric pathogens such as *Aeromonas, Campylobacter, Escherichia coli, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*, parasites, viruses, and other bacteria (Table 32-1).[1,13,18,34,45,47,51,84,87,88,91,97,109,151,171,176,177,182,184] Bottled water has been reported to be the source of the causative agent in cholera,[21,22] and typhoid[27] outbreaks, as well as the diarrheal condition known as "Traveller's Disease."[61,141] Except for these isolated incidents, there have been no major outbreaks involving bottled water. The use of contaminated distilled water in humidifiers has been the cause of asthmatic or respiratory illness, known as "humidifier disease."[177]

During the cholera epidemic of April to November 1974 in Portugal,[21,22] bottled mineral water was identified as one vehicle of transmission of the causative agent *V. cholerae*. The source water from two springs was shown to be contaminated. Although the bottled water itself was not cultured for this bacterium, epidemiological evidence implicated noncarbonated bottled mineral water but not the carbonated version. It was speculated that the source water was contaminated either via a nearby river and/or via sewage from nearby villages; river water may have seeped into the limestone aquifer because the water table was significantly lowered by a lack of rain. Limestone aquifers are notorious for developing fissures and channels through which underground water can flow rapidly without being purified. It was suspected that the initial source of *V. cholerae* was raw or poorly cooked cockles, and the contamination continued through fecal contamination of river waters. The pH of the spring water and noncarbonated bottled water would allow the *V. cholerae* to survive, while in carbonated products this bacterium died within 24 hours. It is not known from the published reports[21,22] if the manufacturing plants tested the bottled waters for bacteria as part of their quality control and assurance. After this outbreak, Portuguese health officials insisted that a deep well be drilled at a higher altitude (away from the river) and that ultraviolet water purification equipment be installed.[22] The bottlers also demonstrated that the well water and finished products of bottled water were free of pathogenic bacteria and safe to drink.

In general, waterborne disease outbreaks are a result of either poor protection of the source water (ground water, well, etc) or inadequate treatment. Microbial contamination of ground water has been responsible for large outbreaks of waterborne diseases, particularly gastroenteritis.[43] Use of contaminated, untreated ground water caused 35% of waterborne disease outbreaks during a 35-year period (1946–1980) in the US[120]; hepatitis A virus, *Shigella, Yersinia,* and *Giardia* species were implicated as the most frequent pathogens. Many intermittent contamination problems are caused by the overflow or seepage of sewage, surface water runoff, and periodic flooding over the well field. New bacterial populations may also colonize an aquifer by filtration from above, as well as by lateral migration.[68] This migration can occur over considerable distances (2 to 3 km) in unconfined sandy aquifers[68] and may be substantial in other ground water, such as granular aquifers where bacteria can be transported several hundred metres or more[74,75]; bacteria and viruses persist longer in ground water than in surface water.[20] Bacteria may be retained by soil and prevented from reaching ground water whereas viruses can pass through the soil reaching the ground water.[20] Viruses have been isolated from ground water flowing beneath landfill and treatment sites for domestic and agriculture sewage.[104]

The appearance of coliform and pathogenic bacteria in water supply wells suggests that some contaminating (nonindigenous) bacteria may survive for considerable lengths of time in ground water.[74] Recent studies of genetically engineered bacteria in aquifers have shown that survival can last from 1 to several months (and up to 1 year), depending upon the bacteria, soil conditions (including chemical contaminants, nutrients and moisture), and competition with indigenous bacteria.[74] *Salmonella typhimurium, Escherichia coli, Streptococcus faecalis,* and poliovirus (type 1) survived longer than 15 days in ground water stored at 22°C[16] while total coliforms, fecal coliforms, and fecal streptococci were reported to survive longer than 70 days in contaminated wells.[19]

32.3 MICROBIOLOGICAL CONTAMINATION OF BOTTLED WATER

In addition to the water source, the most likely sources of contamination are equipment used to pump or transport the water from its source to the bottling location, equipment used in processing or bottling the water, and the bottles and caps used. In an unsanitary manufacturing plant, contaminated bottles and caps can contain up to 10^7 cfu cm^{-2}.[176] Colonization of equipment by bacteria, such as *Pseudomonas aeruginosa,* can occur (Section 32.5.3). Exposure of the water to air and contact with humans and other animals during bottling are additional modes of contamination.[59]

If bottled water, is not produced under Good Manufacturing Practice (GMP), using Hazard Analysis Critical Control Points (HACCP) to manage microbiological hazards, it could be the source of major food poisoning outbreaks. In general, information concerning foodborne illness associated with Canadian domestic and imported bottled water is nonexistent, except for the isolated major outbreaks, already mentioned. No doubt this reflects both the general high quality of most products, and possibly the failure of epidemiologists and physicians to inquire about consumption of bottled water when investigating illness. Another factor may be that many of the bacteria found in bottled water can cause cases of mild, uncomplicated gastroenteritis that are self-limiting. Illnesses such as these are not likely to be reported and even less likely to have the causative agent determined.

Bacteria found in the final product may have the potential for causing disease (Table 32-1).[145,172] Recently, Payment et al.[145] demonstrated a correlation between bacteria released from reverse-osmosis filtration units and gastrointestinal symptoms. Although this study used domestic point-of-use units, the bacteria released are common to all filtration units including those used in the manufacture of bottled water (Table 32-1); therefore the potential for disease exists. These organisms may be secondary or opportunistic pathogens, such as *Acinetobacter, Flavobacterium, Pseudomonas,* and other genera (Table 32-1),[73,94,143,146,176] especially if present in high numbers.[172] Whether individuals who consume contaminated water suffer adverse health effects depends upon the bacteria involved, the number of bacteria ingested, and the individual's general health and resistance to that particular organism.[63] Opportunistic pathogens may cause illness with insidious onset after long incubation periods in immunocompromised persons (see Section 32.4.1).

Bottled water needs to be of high microbiological quality, especially if intended for use by vulnerable populations (pregnant women, the infirm, the elderly, or the very young) for the following reasons. First, up to 50 ml of water, when taken between meals, can pass immediately through the stomach into the intestine. Pathogens in this water escape the bactericidal effect of gastric juices in the stomach, reaching the intestine in the same numbers as initially ingested and subsequently triggering disease.[134] Second, the minimum infective dose of pathogens may be quite low when ingested between meals with limited amounts of water. Third, consumption of bottled water by vulnerable populations, such as the elderly, AIDS patients, HIV-positive individuals, and other immunocompromised persons, will probably increase as these populations increase[5]; in compromised hosts, intragastric bactericidal mechanisms may be impaired to the extent that any microorganism ingested may reach the intestinal tract.[133] It is known that bottled water has been given to hospital patients when there was uncertainty about the purity of tap water.[33] Contaminated water supplies might be a more significant source of infection than foods, especially for infants.[169] In other situations, contaminated water supplies often cause intestinal problems that go unreported because the problems may be short-lived and probably not associated with the ingestion of water.[7] The modes of transmission of bacterial pathogens through water include direct exposure via ingestion or contact with the contaminated water, and indirect exposure through ingestion of foods or products prepared with contaminated water, subsequent contact with infected persons or animals, and exposure to aerosols.[185]

32.4 PRODUCTION OF BOTTLED WATER

Only approved source water (Exhibit 32-1) that has been inspected, analyzed, and found to be safe, sanitary, or potable (by either federal, provincial, regional, or other health authorities) should be used for bottling. This will ensure that the water entering the bottling plant has low bacterial counts and is free from pathogens.

However, during production and distribution of all types of bottled water, the cleanliness or bacterial content of the source water can change dramatically at each step of the manufacturing process and during storage in bottles in the processing plant and on the retailer's shelves.[168] These changes can be brought about by contamination and by growth of the previously stressed, dormant, or starved indigenous microorganisms[168] because of an altered environment (increased temperature, oxygen, and nutrient levels).

At the bottling plant, further microbiological contamination of the water may also occur from equipment used to pump or transport the water from its source to the bottling location, equipment used in the processing or bottling process (such as deionizing columns and filters), as well as from bottles, caps, and environmental sources.[18,59,175,176] A common water treatment system for bottled water (not including "natural" water) may include filtration, demineralization, and purification (Table 32-2, Figure 32-1). Microorganisms present in the source water are trapped in the ion exchange and filtration units, and may proliferate and contaminate the final product if these units are poorly maintained (Table 32-1).[85,145,166,172,174] The microbial quality of water produced by these devices is extremely variable, and is a function of: (1) the contamination and nutrient content of the source water, (2) the service life of the filter, (3) the water temperature, (4) the frequency of static water conditions,[63] and (5) maintenance of the filters. Water from reverse-osmosis filtration units can contain 10^4 to 10^7 cfu ml^{-1},[144] while activated carbon filtration units can release from 10^2 to 10^5 cfu ml^{-1}.[63]

Contamination can occur any time during processing due to failure of hygienic practices.[119] Thus, Hunter and Burge[93] found *Staphylococcus epidermidis* and *S. humanis* in 6 out of 52 bottles of water, and in other studies, bottled water was found to contain coliforms, *S. aureus,* and *Enterococcus.*[162,176] Most of these bacteria can be attributed to poor GMP in the processing plant and possible contamination via plant workers.

However, based upon available information, bottled water sold worldwide by members of the bottled water associations has generally been found to be of good microbiological quality and is not considered to pose any microbiological threat. Bottled water associations, such as the International Bottled Water Association (IBWA) and the Canadian Bottled

Table 32-2 A General Review of Bottled Water Treatment Systems Allowed in North America[a]

Step	*Process*	*Examples*	*Purpose*
1	Source water collection	Artesian well	N/A[b]
		Tap water	N/A
		Glacial melt	N/A
		Springs, etc.	N/A
2	Aeration	—	Removes volatile organics
3	Filtration	Activated carbon filter	Removes solids, odors, organics, and bacteria
		Sand filter	Removes coarse solids
		Manganese filter	Removes sulfur, iron, and solids
4	Demineralization or purification	Water softeners	Removes total dissolved solids, coarse solids, and minerals
		Deionizer	Removes dissolved minerals
		Distiller	Removes dissolved minerals
		Reverse-osmosis filtration	Removes 90% of the dissolved minerals and coarse solids
		Cation, anion, or mixed bed filters	Removes minerals
5	Mineral adjustment	Mineral mix added	Improves taste, mineral composition, chemical (eg, fluoride) composition
6	Disinfection	Ozonation (0.4–0.6 ppm)	Kills bacteria
		Ultraviolet irradiation[c]	Kills bacteria
		Filtration (1–5 μm)	Removes bacteria and parasites[d]
		Carbonation	Lowers pH and kills bacteria
7	Handling of final product	Container filling	N/A
		Capping	N/A
		Coding	N/A
		Distribution	N/A

[a] See references.[17,102,177] NOTE: Some of these processing steps are not allowed for "natural" waters in Europe.[9,37,38]

[b] N/A = not applicable.

[c] >16,000 microwatt seconds cm^{-2} at 254 nm.

[d] For removal of the oocytes of some parasites the filter must be 1 μm.[11]

Water Association (CBWA), issue sound guidance to members on Good Manufacturing Practices for the production of bottled water. All bottlers should be encouraged to join such associations.

Treatment of the source waters is dependent upon the quality of the source water and the type of bottled water being manufactured. Some researchers feel that a minimal treatment for source waters must include filtration for cyst removal, together with disinfection to eliminate pathogenic bacteria and viruses,[63] although this may not be necessary to treat water from protected and approved sources. A filtration system to entrap parasites appears to be the most logical means of removal, provided it is properly operated,[122] and if effective chemical pretreatment (including settling and precipitation of macro or micro particulates) is accomplished prior to filtration.[123] Incorporation of this filtration process must be carefully managed because improper filter backwashing and instability of the filter media can provide opportunities for elution of the cysts into the final product.[63] Subsequent disinfection must be sufficient to kill all cysts; in some cases, the contact time required may be several hours.[86]

32.4.1 Disinfection/Sterilization of Bottled Water

Systems used within the bottled water plant to reduce microbial contamination can include carbonation, ozonation, and ultra-filtration (using a one

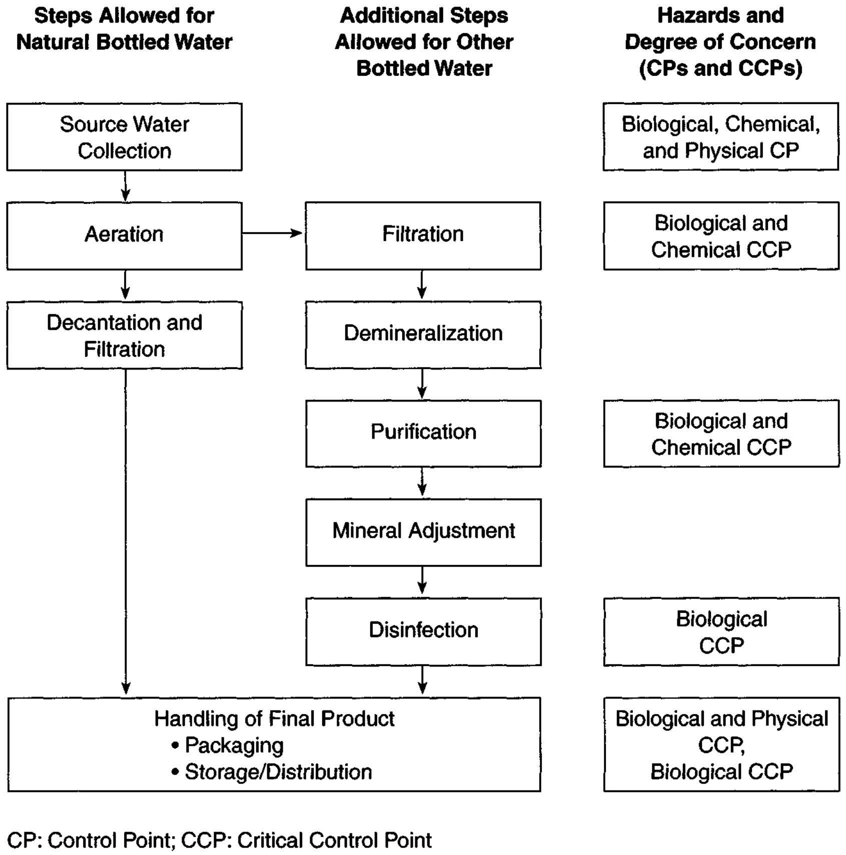

Steps Allowed for Natural Bottled Water
Additional Steps Allowed for Other Bottled Water
Hazards and Degree of Concern (CPs and CCPs)
Source Water Collection
Biological, Chemical, and Physical CP
Aeration
Filtration
Biological and Chemical CCP
Decantation and Filtration
Demineralization
Purification
Biological and Chemical CCP
Mineral Adjustment
Disinfection
Biological CCP
Handling of Final Product
• Packaging
• Storage/Distribution
Biological and Physical CCP, Biological CCP
CP: Control Point; CCP: Critical Control Point

Figure 32-1 Flow Diagram Showing the Manufacturing Processes for Bottled Waters.

micron "Absolute" filter), and other treatments (Table 32-2). The need for a water treatment, including a disinfection system, in a bottled water manufacturing plant is related to the type of bottled water and the intended use.

"Natural" bottled waters (Exhibit 32-1) come from an "approved" protected and sanitary underground source, where contamination from bacteria, parasites, and viruses has been proven to be negligible or nonexistent because of the nature of the surrounding rock formations. By standards and definitions (Exhibit 32-1),[38,39] these bottled waters must contain the indigenous innocuous microflora and therefore cannot be subjected to any process to remove them.[9,38,39,95] However, much controversy surrounds the question of the potential pathogenicity of indigenous microorganisms in mineral waters. Many of these microorganisms may not be pathogenic under normal conditions but have been responsible for infections in special circumstances, such as in hospitalized patients debilitated by illness or treatments that undermine the body's defenses. Some authors advise that such patients should avoid drinking natural mineral waters[168,177] while others recommend these products.[138]

Certain populations or YOPIs (ie, the young, old, pregnant, and immunocompromised) are particularly susceptible to infection by bacterial contaminants and parasites. For this reason, Health Canada recommends that only disinfected bottled water (such as carbonated, ozonated, and UV treated) should be used by these populations.[177]

Similarly, the National Association of People with AIDS (NAPWA) recommends that "safe drinking water sources" include the following: (1) "bottled water that is from a protected source" (75% of bottled water) or (2) "that has been subjected to one of the following: distillation, reverse osmosis, or one micron filtration."[138] Both statements are supported and recommended by the report on a recent workshop dealing with waterborne cryptosporidiosis.[11] Although when discussing protected sources, this report states that "water from underground sources is unlikely to contain oocytes, if it is protected from possible contamination that results from intermittent mixing with surface water, and it has been consistently free of coliform bacteria when tested."[11] The use of a one micron "Absolute" filter for the removal of protozoan cysts and the other purification steps is consistent with Health Canada's recommendation for disinfection of bottled waters targeted for susceptible consumers.

In contrast, the UK Expert Advisory Group on AIDS[12] recommends that AIDS patients with CD4 cell counts below 200 mm^{-3} be advised to boil water (from any source) before drinking it, as a measure to prevent waterborne cryptosporidiosis. Persons with asymptomatic HIV infection and/or CD4 cell counts above 200 mm^{-3} should be apprised of the risks of cryptosporidiosis so they can make an informed decision about boiling water. Physicians treating other immunosuppressed patients should consider the need to advise patients to boil water, depending on the severity of the immunodeficiency and the likelihood that cryptosporidiosis, if contracted, would be severe and persistent.

As a means of controlling the bacterial content (aerobic colony count, ACC) and the normal proliferation of indigenous and contaminating bacteria in bottled water (not "natural" products), water bottlers should be encouraged to add a final disinfection step, such as carbonation or ozonation, to their manufacturing process. Adequate disinfection can be the most cost-effective public health insurance available provided the treatment reduces all pathogenic bacteria by at least 4 logs[62]; assuming that the initial bacterial load is low.

The pH of the bottled water can affect the bacterial growth in these products and the pH may be significantly lowered by carbonation of the water, which is not unexpected due to the documented antimicrobial action of carbon dioxide.[92] In one study, noncarbonated mineral water had a pH of 6.2 to 6.5, while the pH of carbonated mineral water was 4.9 to 5.1[25]; enteropathogenic bacteria declined more rapidly in carbonated water because of the lower pH and bactericidal effect of the CO_2.[25] Carbonated products sampled in Canadian studies had 0% to 4% rejection rates as a result of ACC, compared with 61% for other types of nontreated bottled water.[176] Similarly, Ruskin et al.[156] found the lowest ACC to be in sparkling or carbonated products sampled in the US Virgin Islands. Hunter[92] reported that in several studies only 3% to 8% of carbonated waters studied had ACCs >100 cfu ml^{-1}. In contrast, more than 52% (and up to 72%) of the still waters tested had ACCs >1,000 cfu ml^{-1}.[92] There is no published literature on flavored bottled waters. However, most sold in Canada have been found to be high in carbonation and would have the bactericidal effects of low pH and high CO_2.

Ozonation has also been shown to reduce counts substantially, retard bacterial growth, disinfect, and even sterilize the product.[176] Ozonation of some finished products is allowed by the bottled water associations and need is dependent upon the quality of the source water and the type of bottled water it will be marketed as, ie, "natural" bottled water cannot be ozonated while other bottled waters can be (Exhibit 32-1; IBWA and CBWA, personal communication).

Refrigerated storage will minimize bacterial multiplication in bottled water, and the consumer should be advised to keep bottled water refrigerated after opening,[6,66,156,176] especially those products that may be consumed directly from the bottle and stored between use. This will help deter growth of contaminants that are introduced from air entering the bottle upon opening and those introduced from oral contact. Water dispensers (coolers) that utilize the larger bottles should be equipped with refrigeration units.

32.5 THE EFFECT OF STORAGE ON THE MICROBIOLOGY OF BOTTLED WATER

32.5.1 Indigenous Bacteria

Indigenous bacteria remain at low numbers while the water is in its natural environment but commence rapid growth after the water is bottled.[131,176] Prolonged storage of most waters at room and refrigeration temperatures allows multiplication of both indigenous and contaminating bacteria to numbers $>10^4$ or 10^5 cfu ml^{-1}.[18,50,58,66,131,159,176] Some bottled mineral waters do not support bacterial growth (Warburton, unpublished data), which may reflect their high mineral content and the high osmotic pressure this would exert. The reasons for this altered growth include increased oxygenation of the water during the bottling process, the increased surface area provided by the bottle, trace amounts of nutrients arising from the bottle,[159] and the increased temperature of the bottled water compared with the source water.

Secondary growth may occur as some species die, autolyze, and provide nutrients for the growth of other species, such as heterotrophic enteric pathogens.[133,159] Even small numbers of potentially harmful bacteria can survive for long periods in noncarbonated water, and multiply, feeding on the products of lysis, or on the metabolites of autotrophs. Survival curves with a saw-toothed appearance are often typical of bacterial growth in water,[71] and this appearance is probably indicative of a repeating cycle of bacterial multiplication, followed by die-off resulting from a shortage of nutrients, then cell lysis, subsequent release of nutrients, and another round of multiplication[133]; although in some cases, the overall bacterial population slowly decreases over time. Long-term changes in the major heterotrophic bacterial populations of bottled water occur, which may reflect changes in nutrient availability, a decrease in viability of some strains, competition, antagonism, synergism, or other factors not yet understood.[131,179,180] Bacterial multiplication is more rapid and reaches higher numbers in bottled water stored in plastic containers than in those stored in glass.[50] This is probably due to chemical components or substances migrating or leaching from the container walls into the water. Other influences may include oxygen and other gases diffusing through the plastic containers into the water.

During storage at ambient temperatures in plastic and glass containers, growth commences 1 to 2 days after bottling and may result in populations of 10^5 to 10^6 cfu ml^{-1}.[140,176] Counts may occasionally exceed 10^7 cfu ml^{-1}. These results are consistent with other studies, where bacterial populations in commercial samples (10^3 to 10^6 cfu ml^{-1}) were higher than those found in water collected from the spring (10^2 to 10^3 cfu ml^{-1}).[70]

In a 1987–1988 Canadian survey of bottled water,[176] sample units representing 82 lots of bottled water were obtained from 40 manufacturers in Halifax, Montreal, Toronto, Winnipeg, and Vancouver regions. Approximately half of the sample units (410 in total, usually 5 per lot) were analyzed within 1 day of bottling while the other half (395 sample units) were stored at room temperature and analyzed after 30 days. At day 0, 91% of the bottled water had ACCs $<10^4$ ml^{-1}, while only sample units of distilled and glacial waters had ACCs $>10^5$ ml^{-1}. All samples of drinking, deionized, unfiltered, mineral, and spring waters had an ACC <100 ml^{-1}. After 30 days of storage, only a few sample units of distilled water had very high ACCs ($>10^6$ ml^{-1}) while the number of sample units of distilled, demineralized, treated, and bottled waters with ACCs between 10^2 and 10^6 ml^{-1} had increased by 30%. Throughout this study, coliforms, *Aeromonas* spp., and *Pseudomonas* spp. were not detected.

A variety of surveys, including routine monitoring/sampling of spring, mineral, and other bottled waters,[176] show a wide range of bacterial counts (ACCs) in these products. Generally, spring and mineral waters had lower ACC levels than did other bottled water. Out of the varieties of bottled water tested, distilled water tended to have higher ACCs and also contained coliforms and *Pseudomonas aeruginosa*.[176] In contrast, glacial and deionized waters had lower ACCs than did some water classified as "bottled or purified" waters.

32.5.2 Inoculation Studies

Studies have been conducted to determine the effects of postprocessing contamination, poor GMP/HACCP, and storage on the survival of contaminating bacteria and subsequently the quality of bottled water (Table 32-3). When low levels of bacteria (≤ 100 cfu ml^{-1}) were inoculated into bottled mineral and spring water (simulating low contamination), their numbers increased to 10^3 to 10^6 within 1 week (Warburton[181] and unpublished data). *Aeromonas hydrophila, Citrobacter freundii, E. coli* O157, and *P. aeruginosa* reached levels $>10^3$ cfu ml^{-1} within 2 days while *Salmonella typhimurium, S. senftenberg,* and *Serratia marcescens* multiplied more slowly, taking 5 to 6 days to reach these levels. Thus, pathogens can proliferate to harmful levels in bottled water even when the initial contamination levels are low.

Other inoculation studies were done with high levels of bacteria to simulate gross contamination of the source waters and final products by sewage or surface waters as well as extremely poor GMP in the manufacturing plant.[179–181] The latter could occur, for example, during filtration and purification steps (Table 32-2) when resin filters and activated carbon filters become overloaded and release concentrated nutrients and bacteria into the final product.[179] A variety of bacteria, including *Aeromonas hydrophila* and other pathogens, can proliferate and survive in inoculated bottled water stored for more than several weeks (Table 32-3 and Figure 32-2). When initially present in high levels, *A. hydrophila* could survive up to 60 days or longer in water stored at 22°C. The presence of other bacteria, such as *E. coli, Lactobacillus, Flavobacterium,* and especially *P. aeruginosa*, may enhance the survival of *Aeromonas* in bottled water.[179]

Warburton et al.[180] also studied the survival of *P. aeruginosa* and *Salmonella* when coinoculated into distilled water and other bottled water (Table 32-3). After inoculation in distilled water, the nutrient levels were sufficient to allow some growth (approximately 1 log) and subsequent survival of both *P.*

Table 32-3 The Survival of Bacteria in Bottled Water

Bacteria	*Water Type*	*Bottle Type*[a]	*Inoculation Level*[b]	*Competitor Level*[c]	*Survival (days)*	*Reference*[d]
Aeromonas hydrophila	distilled	P	5.40	NA	37	179
	distilled	P	5.40	NA	41	179
	distilled	P	4.85	5.0 PA	63	179
	distilled	P	5.48	5.0 LAC/FLA	50	179
	distilled	P	5.70	6.0 EC	45	179
	mineral	NR	4.00	ND	70	25
	mineral	P	Natural Contam.	ND	69	163
Clostridium perfringens (vegetative cells)	spring	P (21°C)	5.48	ND	7–10	UN
	spring	P (37°C)	5.70	ND	10–14	UN
	spring	P (21°C)	4.90	ND	10–14	UN
	spring	P (37°C)	5.08	ND	7–10	UN
	spring	P (21°C)	3.78	ND	7–10	UN
	spring	P (37°C)	5.04	ND	7–10	UN
	mineral	P (21°C)	5.65	ND	31–35	UN
	mineral	P (37°C)	5.60	ND	7–10	UN
Enterobacter cloacae	mineral	P	5.43	5.04–6.01 ACC-I	>20	132
	mineral	P	4.75	NA	>20	132
	mineral	G	4.75	NA	>20	132
	tap	G	4.75	NA	>20	132
Escherichia coli	mineral	NR	4.00	?	42	25
	mineral	P	4.35	NA	20	132
	mineral	P	4.35	5.04–6.01 ACC-I	>20	132
	mineral	G	4.50	NA	>20	132
	tap	G	4.35	NA	>20	132
E. coli O157	distilled	G	5.42	NA	>309	181
	mineral	P	5.45	NA	>309	181
	mineral	P	6.54	NA	>264	181
	mineral	P	1.0	2.7 PA, 3.0 ACC-A	22–36	181
	mineral	P	4.16	NA	>44	181
	river	P	4.45	2.5 ACC-I	24–44	181
	river	P	5.75	2.5 ACC-I	35-42	181
	spring	P	4.20	3.0 PA	>48	181
	spring	G	4.20	NA	>48	181
	spring	P	3.00	3.0 ACC-I	74–94	181
	spring	G	3.24	NA	>102	181
	spring	P	3.74	3.0 ACC-I	53–73	181
	spring	P	3.30	2.6 PA, 3.0 ACC-A	34–49	181
	spring	P	2.04	1.0 PA, 2.7 ACC-A	>20	181
	tap	G	4.72	NA	>303	181
	well	P	3.48	2.3 PA, 3.0 ACC-I	58–65	181

continues

Table 32-3 Continued

Bacteria	*Water Type*	*Bottle Type*[a]	*Inoculation Level*[b]	*Competitor Level*[c]	*Survival (days)*	*Reference*[d]
Klebsiella pneumoniae	mineral	P	5.20	NA	>20	132
	mineral	G	5.35	NA	>20	132
	mineral	P	4.80	5.04–6.01 ACC-I	>20	132
	tap	G	5.20	NA	>20	132
Pseudomonas spp.	mineral				>140	190
P. aeruginosa	mineral	NR	4.00	ND	70	25
	distilled	NR	5.45	NA	128	180
	distilled	NR	6.00	6.15 SS	>135	180
	distilled	NR	5.95	5.38 SAL	>139	180
	distilled	NR	6.15	5.90 SAL	>140	180
	distilled	NR	6.13	6.11 SAL	145	180
	spring	NR	6.08	6.30 SAL	>137	180
	spring	NR	6.43	6.40 SAL	>138	180
	mineral	NR	6.41	6.30 SAL	>135	180
	mineral	NR	6.45	6.15 SS	>138	180
	mineral	NR (6°C)	1.99	4.50 ACC-I	210	71
	mineral	NR (20°C)	2.10	5.10 ACC-I	>365	71
	mineral	NR (30°C)	2.10	4.45 ACC-I	>365	71
	mineral	NR (37°C)	2.25	4.45 ACC-I	>365	71
	mineral	G	5.40	NA	>20	132
	mineral	P	5.38	5.04–6.01 ACC-I	>20	132
	mineral	P	5.80	NA	>20	132
	tap	G	5.50	NA	>20	132
	tap	NR	5.30	5.00 SS	>136	180
	tap	NR	5.30	5.30 SAL	>138	180
	well	NR	6.48	6.00 SAL	>96	180
	well	NR	6.30	6.00 SAL	>96	180
	well	NR	5.85	NA	108	180
	well	NR	6.48	6.00 SAL	>138	180
	well	NR	6.30	6.00 SAL	>138	180
	well	NR	5.95	4.60 SS	144	180
Salmonella senftenberg	mineral	NR	6.15	6.45 PA	>162	180
	distilled	NR	5.95	NA	>158	180
	distilled	NR	6.15	5.00 PA	>159	180
	tap	NR	5.00	5.30 PA	>136	180
	well	NR	4.60	5.95 PA	8–12	180
Salmonella typhimurium	mineral	NR	4.00	ND	70	25
Salmonella spp.	distilled	NR	6.38	NA	100–119	180
	distilled	NR	5.48	NA	67–75	UN
	distilled	NR	5.38	5.95 PA	>139	180
	distilled	NR	5.90	6.15 PA	>140	180
	distilled	NR	6.11	6.13 PA	>169	180
	spring	NR	6.15	NA	>137	180
	spring	NR	6.30	6.08 PA	>137	180
	spring	NR	6.27	NA	>162	180
	spring	NR	6.40	6.43 PA	>162	180

continues

Table 32-3 Continued

Bacteria	*Water Type*	*Bottle Type*[a]	*Inoculation Level*[b]	*Competitor Level*[c]	*Survival (days)*	*Reference*[d]
Salmonella spp. (continued)	mineral	NR	6.30	6.41 PA	>135	180
	tap	NR	5.30	5.30 PA	>138	180
	well	NR	6.00	6.48 PA	63–96	180
	well	NR	6.00	6.30 PA	63–96	180
	well	NR	6.00	6.48 PA	>138	180
	well	NR	6.00	6.30 PA	>138	180

[a] Bottle type: G: glass bottle; P: plastic bottle; NR: not reported; (°C): storage temperature.

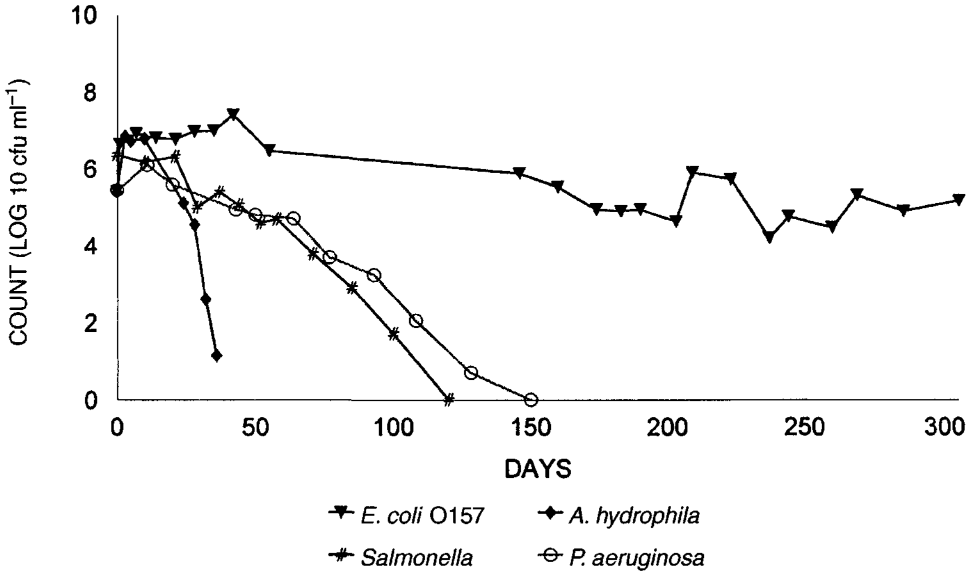
[b] Counts: log cfu ml^{-1}.

[c] NA: not applicable (sterilized sample); ND: not determined; PA: *P. aeruginosa* added; ACC-I: indigenous aerobic colony count; ACC-A: aerobic bacteria added; LAC/FLA: *Lactobacillus* and *Flavobacterium*; EC: *E. coli*; SS: *S. senftenberg*; SAL: *Salmonella* spp.

[d] UN: Warburton (unpublished data).

aeruginosa and *Salmonella* at high levels for up to 60 days; after this time, the bacteria quickly decreased in numbers, probably because of nutrient depletion. It is well known that enteric bacteria become stressed by nutrient limitations in aquatic systems, especially where organic material is persistently low.[130] The nutrients added along with the inoculum were likely to be insufficient to result in a pH change or allow the creation of toxic metabolites, so these are not likely reasons for the die-off. In these studies,[180] *P. aeruginosa* survived as the sole contaminant in distilled water for approximately 128 days. Alone, *Salmonella* survived in distilled water for 100 to 119 days. Together, both bacteria survived longer (>145 days). This seems to indicate that there is a synergistic effect between the two bacteria. Alone, *S. senftenberg* survived in distilled water for >158 days. Being often more resistant to heat and destruction by alcohol than other *Salmonella* serotypes,[178] it was speculated that this serotype may survive longer under these stressful conditions. It did so by at least 40 days.

Washing the cells before inoculation resulted in a less dramatic decrease in number compared with



These data parallel data for other types of bottled water (Table 32-3)

Figure 32-2 Survival of *Aeromonas hydrophila, Pseudomonas aeruginosa,* salmonellae, and *Escherichia coli* following inoculation into sterile distilled water stored at 22°C.[179-181]

unwashed cells,[180] and the former showed a prolonged survival time. Initially, after inoculation into the distilled water, the counts of both bacteria dropped 1 to 2 logs, but over several days the counts increased approximately 1 log, with both bacteria subsequently gradually decreasing in numbers. Then, in contrast to the *Salmonella*, the *P. aeruginosa* increased in numbers as the *Salmonella* continued to gradually die off. The increase in *P. aeruginosa* was probably due to a small but significant release of nutrients from the *Salmonella* cells. Subsequently, as the *P. aeruginosa* increased in numbers, the *Salmonella* began to increase, although with at least a 20-day lag. Washed cells of both bacteria survived >140 days.

The removal of nutrients from the washed cells may have resulted in a reduced metabolism and inhibited growth, and caused the cells to become dormant, thus prolonging their survival. Miniaturization of marine bacteria and decreased physiological activity are known responses to nutrient limitations that appear to extend their viability with time.[130] Many enteric bacteria are able to carry on a maintenance level of metabolism by hydrolyzing endogenous macromolecular reserves.[130] In contrast, the presence of nutrients, albeit low in concentration, possibly kept the unwashed cells in a state of active growth and more susceptible to die-off when nutrients became scarce.

The type of water (and thus the nutrient content) can influence the survival of bacteria (Table 32-3). *Pseudomonas aeruginosa* alone survived in distilled water for 128 days.[180] Alone, *Salmonella* survived in distilled water for 100 to 119 days, and in natural spring water for 116 to 162 days. When co-inoculated, both bacteria survived longer in the other varieties of water; the exception being the well water. Both *Salmonella* and *P. aeruginosa* survived longer than 135 days in spring, mineral, and tap water. Generally, these waters have a high mineral content (including trace nutrients) and could support bacterial growth for long periods of time as opposed to the inoculated distilled water. Other trials used the same combination of pooled cultures of both bacteria in five different bottled waters and these results show the effect of the water type on the survival of the bacteria. Again, the general trend is that when both bacteria are present, there is a synergistic effect, resulting in longer survival.

Previous studies[179] showed that *P. aeruginosa* enhanced the survival of *A. hydrophila*, which could survive up to 60 days or longer in water stored at 22°C, while *Salmonella* (in the presence of *A. hydrophila*) proliferated and survived up to 70 days. Burge and Hunter[25] also found that *S. typhimurium* was able to persist in bottled mineral water for at least 70 days.

Similarly, *Pseudomonas* spp. survived more than 140 days in mineral water bottled in both plastic and glass bottles.[190] *P. aeruginosa* survived in bottled mineral water for at least 70 days.[25] In contrast, Gonzalez et al.[70] found that *P. aeruginosa* survived and multiplied in mineral water, reaching values greater than 10^4 bacteria ml^{-1} and surviving longer than 1 year. At the conclusion of these studies, *P. aeruginosa* was called a "permanent contaminant" because of its ability to survive "ad infinitum."

The survival curves for *P. aeruginosa* published by Gonzalez et al.[71] are quite dramatic, showing large changes in growth and die-off, possibly because a single strain was used. Warburton et al.[180] used a pool of 10 *P. aeruginosa* strains and observed slight fluctuations between growth and die-off in the survival curves. Similarly, a greater fluctuation was seen when the survival curve of a single strain of *A. hydrophila* was plotted, as opposed to that of a pool of strains of this bacterium.[180]

In contrast, vegetative cells of *Clostridium perfringens* could not survive in a variety of types of bottled water even when inoculated at levels > log 3.0 (Table 32-3; Warburton, unpublished data). This bacterium did not multiply in any sample of bottled spring and mineral waters, decreased in counts by 3 to 4 logs within 1 week, and most strains completely died off within 2 weeks. One strain inoculated into mineral water and another strain inoculated into spring water (both stored at 21°C) survived longer than 2 weeks at counts < log 1.0 (after dropping > 3 logs within 1 week). Temperature abuse (storage at 37°C) did not affect the survival rate nor did it stimulate multiplication.

32.5.3 Biofilm Formation

In bottled water manufacturing plants, biofilms can play an important role in the survival of contaminating bacteria in pipes, filters, water reservoirs, and in the finished products. The proportion of bacteria adhering to surfaces is usually much greater than the planktonic population in the bulk fluid. Indeed, the majority of bacteria in aquatic systems grow within an expolysaccharide enclosed matrix.[41,121] Contamination of water from biofilms adherent to container walls may occur as cells are gradually released, or as large fragments of biofilm become detached from the walls.

Several authors have found higher counts and faster growth in bottled water stored in plastic or PVC bottles than in glass bottles,[18,50,140] however, this may be dependent upon the storage temperature.[70] At 22°C, bacterial growth was more rapid and reached higher numbers in mineral water stored in plastic bottles, while at 37°C growth was higher in glass bottles.[70] In contrast, De Felip et al.[48] demonstrated higher bacterial populations in mineral water stored at 20°C and 37°C in PVC, than in Tetrabrik containers and glass bottles (in that order). The inner surfaces of plastic bottles are rougher than that of glass, thereby promoting adhesion and colonization.[30] Also, residual cleaning agents in glass bottles (more so in recycled bottles) may inhibit bacterial growth.[18,30] The ability to adhere to plastic walls allows bacteria to utilize the few chemical components and organic sources that migrate to solid surfaces as well as the fatty acids, which may be liberated by lipase activity from the plasticizers.[50,73,159] Diffusion of gases through the walls of the plastic containers will aid bacterial growth in these bottles.

The colonization of surfaces enhances the capacity of bacteria to resist disinfection and starvation.[132] For example, *P. aeruginosa* had a lower mortality rate in water bottled in PVC than glass, which may reflect the capacity of this bacterium to colonize this type of material.[132] In contrast, the type of container did not influence the survival of enterobacteria.[132]

Long-term survival of bacteria in bottled water may be due in part to biofilm formation on the walls of the container.[173] Heterotrophs, *A. hydrophila,* and *E. coli* were able to survive in biofilms at levels of over 1,000 cfu cm^{-2} and higher for >21 days.[173] There was a fluctuation in the biofilm and planktonic counts,[173] which no doubt is a reflection of the continual incorporation of cells into the biofilm, sloughing off of cells into the liquid, as well as multiplication/die-off of the bacteria. Viable *E. coli* O157 was recovered more than 300 days after inoculation into sterile tap water.[181] Examination of the container by scanning electron microscopy demonstrated biofilms of *E. coli* O157, which had formed on the container walls. The cells were encased in exocellular material, probably exopolysaccharide. Large areas of the surface demonstrated empty cell wall sacculi, which remained after death and lysis of the biofilm cells. The contents released from the lysed cells may have provided nutrients for surviving cells to grow (cryptic or cadaveric growth) and form new biofilms.[133] Biofilms formed by *E. coli* O157 in nutrient-rich (tryptic soy broth) conditions[181] resulted in extensive adherence within 24 hours, with extracellular material quickly forming and increasing to levels that obscured individual cells. *Aeromonas, E. coli,* and other pathogens can become incorporated into biofilm communities, thus enhancing their viability.[173]

Some bottled water is low in available nutrients, and may be considered an oligotrophic environment. Bacterial biofilms on container walls probably form very slowly over a period of days or weeks. Warburton et al.[181] found sparse adherence and poor formation of biofilms by *E. coli* O157 in nutrient-limited (distilled water) after 10 days, although biofilms were present after 300 days.

32.5.4 Contamination of Water Coolers

In a 1992 study of water coolers in Quebec City, 28% to 36% of the water samples from these dispensers was found to be contaminated with coliforms, fecal coliforms, fecal streptococci, *Staphylococcus aureus,* or *Aeromonas* spp.[118] This contamination was not related to GMP of the production of the water in the bottled water plant but to the use of water coolers as the dispensers for the water.[118]

Whereas contamination of a water cooler by the bottled water supply can be controlled during production, the environment to which the water bottles and dispensers are exposed cannot be strictly controlled because there exists the possibility of contamination from the air and use.[54] The greatest potential for microbial contamination of bottled water occurs during bottle changing of water dispensers.[54] There is the possibility of contaminating the water in the dispenser with skin bacteria from the handling of the external bottle surfaces[54]; also, the reservoir is open to the air at this time. With some dispensers, the contamination route is the metal shaft used to puncture the caps to allow the water to drain into the closed reservoir.[54] If contaminants, such as *E. coli* or other bacteria, are on the bottle cap or the recessed plastic channel used to guide the neck of the bottle onto the metal puncturing shaft, then these bacteria will eventually be smeared across the shaft, or possibly migrate in any residual water, when pulling empty bottles out during bottle changes.[54]

Regular cleaning of water coolers and disinfection with hypochlorite should limit contamination.[118] In the 1992 study in Quebec City, only 44% of people possessing water coolers in residences and 36% of personnel possessing water coolers in workplaces had been informed of the necessity to clean and disinfect the equipment, let alone how to do it.[118] Because of this lack of information, a variety of products (including baking soda, vinegar, and dishwashing liquid) were used, rather than hypochlorite. Similarly, in a 1993–1994 survey of 48 Calgary schools using water coolers 80% to 90% never received any information on cleaning and maintenance procedures (K.G. Orom, Calgary Board of Education, personal communication). It is imperative that vendors and suppliers of water dispensers impress upon their clients the need for, and methods of, regular maintenance of their equipment[118] (K.G. Orom and personal communication).

32.6 MICROBIOLOGICAL TESTING AND CRITERIA FOR BOTTLED WATER

The examination of bottled water for indicators and some pathogens is essential after bottling.[116] Rather than examining water for the presence of all the possible pathogens using a variety of technically difficult, insensitive, time-consuming, and expensive tests, it is better to utilize relatively simple and more rapid procedures such as the detection and enumeration of indicator organisms.[168,171] Table 32-4 lists a variety of these indicator organisms that should be tested for, as suggested by the FAO/WHO Codex Alimentarius Commission,[37–40] the European Union,[9,10] Health Canada,[4] and the US FDA.[60] The presence of fecal indicators, such as coliforms, *E. coli*, streptococci, and clostridia (some of which may also be pathogenic) suggests that the water is potentially dangerous and was manufactured under poor GMP. One study found that the presence of indicator bacteria (at any level, even at very low levels of <10 cfu ml^{-1}) was associated with acute gastrointestinal disease.[192] The absence of fecal indicators is taken to denote the absence of bacterial pathogens, in general, and to indicate the product as being safe. The rationale for this is that pathogens are greatly outnumbered in feces by normal intestinal microorganisms.[168] The reliability of coliforms as fecal indicators is discussed in Section 32.6.2.

In Canada, bottled water is legally defined as a "food," although it has also been advertised for use in reconstituting baby and other foods, as nursery drinking water, for cleaning contact lenses, for skin care, and for filling humidifiers. Consequently, when offered for sale to the consumer, it must comply with all provisions of the Canadian Food and Drugs Act and Regulations.[4] Included in these provisions, for example, is Section 4 of the act that states that "no person shall sell an article of food that (i) has in or upon it any poisonous or harmful substance; (ii) is unfit for human consumption; (iii) consists in whole or in part of any filthy, putrid, disgusting, rotten, decomposed or diseased animal or vegetable substance; (iv) is adulterated; or (v) was manufactured, prepared, preserved, packaged, or stored under unsanitary conditions."

Despite the fact that bottled water is considered a food, some producers persist in recommending bottled water as suitable for cleaning contact lenses, reconstitution of rehydration mixtures, and the preparation of pharmaceutical agents. Water used in pharmaceutical preparations and drugs, and as a medical device, must meet the standards established by the regulatory agencies of each country for these products.

The standards for aerobic colony count (ACC), coliforms, and other bacteria are often expressed in terms of a 3-class attribute acceptance sampling plan, commonly referred to as a 3-class sampling plan.[89,90,96,175,176] The recommended parameters for bottled water are shown in Table 32-4, and the reasons for the standards are discussed in the following sections.

32.6.1 Aerobic Bacteria (ACC)

In surveillance programs conducted by Health Canada between 1981 and 1989, noncompliance of bottled water (noncarbonated and nonozonated) with the ACC standard[82,83] was generally quite high and ranged from 19% to >30%[175,176]; the incidence of noncompliance of bottled water with the coliform standard was lower (2% to 3%) but still significant.[176] These results parallel the noncompliance found in other Canadian studies[162,176] and in American studies.[7] All domestic carbonated mineral water samples were in compliance with the ACC standards. Since the ACC counts increase when the product is held at room temperature,[176] there is a subsequent increase over time in the number of lots out of compliance.

Despite the controversy over the effects of ACC on health (see Section 32.3), some authors[49,155,159] question applying an ACC standard to the final product for the following reasons.

1. Multiplication of bacteria in bottled water of low nutrient content is normal.
2. The ACC is not always an indicator of pollution.
3. The number of microorganisms per ml may not be an indicator of microbiological quality or healthfulness.
4. Indigenous bacteria may impute beneficial medicinal and therapeutic properties.

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Table 32-4 Present Regulations, Proposed Regulations, and Microbiological Limits for Bottled Water at the Source and at Other Critical Control Points

Country and Microorganism	*n*	*c*	*m*	*M*
A. Canada[a,4,177]				
All Bottled Water				
ACC	5	2	10^2 ml^{-1}	10^4 m^{-1}
coliforms	10 (5)	1	0 100 ml^{-1}	10 100 ml^{-1}
Aeromonas hydrophila	5	0	0 100 ml^{-1}	
Pseudomonas aeruginosa	5	0	0 100 ml^{-1}	
fecal streptococci[b]	5	0	0 100 ml^{-1}	
spore-forming sulfite-reducing anaerobes (clostridia)[b]	5	0	0 100 ml^{-1}	
Escherichia coli[b]	5	0	0 100 ml^{-1}	
parasites	5	0	0 100 ml^{-1}	
B. Codex Alimentarius Commission[37–40]				
Natural Mineral Water				
coliforms	5	1	0 250 ml^{-1}	2 250 ml^{-1}
E. coli	5	0	0 250 ml^{-1}	
fecal streptococci	5	1	0 250 ml^{-1}	2 250 ml^{-1}
spore-forming sulfite-reducing anaerobes (clostridia)	5	1	0 250 ml^{-1}	2 250 ml^{-1}
P. aeruginosa	5	0	0 250 ml^{-1}	
Bottled Drinking Water[c]				
Draft standard for *E. coli* (April 1999)	?[d]	0	0 100 ml^{-1}	
C. European Community[9,10]				
Natural Mineral Water				
ACC within 12 hours after bottling	?[d]	0	10^2 ml^{-1} [e]	
		0	20 ml^{-1} [f]	
ACC at source	?	0	20 ml^{-1} [e]	
		0	5 ml^{-1} [f]	
coliforms	?	0	0 250 ml^{-1}	
E. coli	?	0	0 250 ml^{-1}	
fecal streptococci	?	0	0 250 ml^{-1}	
P. aeruginosa	?	0	0 250 ml^{-1}	
spore-forming sulfite-reducing anaerobes (clostridia)	?	0	0 50 ml^{-1}	
Bottled Drinking Water				
ACC at 22°C[g]	?	0	10^2 ml^{-1}	
ACC at 37°C[g]	?	0	20 ml^{-1}	
Total and fecal coliforms, fecal streptococci, *P. aeruginosa*[g] sulfite-reducing clostridia[g]	?	0	0 250 ml^{-1}	
D. United States (FDA)[42,60]				
Bottled Water				
coliforms by MPN	10	1	2.2 100 ml^{-1}	9.2 100 ml^{-1}
coliforms by membrane filter	10	1	1 100 ml^{-1}	4 100 ml^{-1}

[a] ACC standards should be applied to all bottled water within 24 hours of packaging. The number of sample units analyzed for coliforms is now 10, but will be changed to 5. *A. hydrophila* and *P. aeruginosa* regulations are in the process of being implemented through legislation.

[b] These guidelines are based on those of Codex[37] for the source water and critical control points.

[c] Bottled Drinking Waters (other than Natural Mineral Water) must meet public drinking water standards if obtained from a public water system or must be of safe and suitable quality (ie, potable) using multiple barrier treatments (filtration, disinfection, etc). Specifications for specific bacteria are under development.

[d] The number of sample units is not specified.

[e] Determined at 20–22°C in 72 hours on agar.

[f] Determined at 37°C in 24 hours on agar.

[g] Determined using ISO (International Standards Organization) methods.

Note: *n* = The number of sample units, usually selected at random, from a lot and examined in order to satisfy the requirements of a plan.

c = The maximum allowable number of marginally acceptable sample units per lot, ie, the number of sample units allowed with counts between *m* and *M*.

m = The maximum number of microorganisms per unit (ml) that is of no concern or is an acceptable level.

M = The number of bacteria per unit that indicate a potential health hazard, imminent spoilage, or gross insanitation, and if exceeded in any one sample unit causes rejection of the lot in question.

5. "Natural" bottled waters (Exhibit 32-1) are sold with the understanding and legal requirement that they have not been subjected to any treatment that would remove natural indigenous bacteria,[9,38,39] as these bacteria are believed to have therapeutic qualities.[59,70]
6. It has never been proven, despite the wide use of mineral waters, that indigenous microorganisms at the levels encountered have had any adverse effect on health.[49,159] Yet, susceptible populations are warned to avoid drinking natural mineral waters.[168]

However, ACC can be used as a means of verification of HACCP and GMP in bottled water plants. High ACC values may indicate poor GMP during the processing of the bottled water, especially when the ACC is determined within 24 hours of bottling, ie, before normal growth of indigenous bacteria occurs. A sudden increase in the ACC from ground water sources may be an early sign of pollution of the aquifer, and is useful in assessing the efficacy of the water treatment processes, such as filtration and disinfection, and as a measure of quality deterioration in distribution lines and reservoirs.[81] Thus, ACC can be used for quality control in the bottling plants. The ACC may be used to determine the suitability of the bottled water for use in the manufacture of other food and drink where, to minimize spoilage, the numbers must be low.[184,185]

Because ACC is more of an indication of GMP (rather than health and safety, which is determined by other indicators), Health Canada is recommending that the standards for ACC in bottled water be removed from regulations and that the ACC standard be incorporated by the bottled water industry into an industry-regulated code of practice, and that bottled water manufacturers be certified by government inspectors. Subsequently, when bottled water is tested for ACC within 24 hours of packaging and found to have excessive ACC, the cause or source of contamination should be investigated (by the quality control manager and health agencies), with identification of some of the contaminants to ensure public safety. Corrective action would then be taken by the manufacturer to improve the final product and when needed (ie, public safety is compromised) public recall or notification would occur.

High ACC numbers in bottled water may decrease the sensitivity of the coliform test,[66] and mask the presence of pathogens and opportunistic (secondary) bacteria.[125] They also increase the risk of exposure of susceptible populations, ie, the infirm, the elderly, and the young, to large numbers of opportunistic (secondary) pathogens.[65,66,145,157]

High ACC values may not indicate fecal pollution. However, when determined at higher temperatures such as 35°C on a rich medium, the ACC is more likely to recover fecal microorganisms that have contaminated the waters,[165,168] as such counts will be low or absent in waters of good microbiological quality.[115,116] Payment et al.[145] have shown that there is a strong correlation between water containing high ACC (obtained at 35°C) and gastrointestinal symptoms. ACC standards should be consistent and uniform for all types of bottled water (Table 32-4).

32.6.2 Coliforms

Coliform contamination may indicate a fluctuation of the source water quality, poor sanitation practices (or GMP) in the plant, and a loss of barrier protection of the sealed containers (ie, postprocessing contamination).[63,81] The absence of coliforms is generally believed to signify a safe drinking supply because pathogenic bacteria, such as *Vibrio cholerae, S. typhi,* and other *Salmonella*, die off at a much faster rate than coliforms.[129] However, waterborne illness epidemics have been reported in which pathogens have been isolated from waters that would have been considered safe by coliform standards.[52] The presence of opportunistic pathogens[94,146] in spring, mineral, and bottled water, as well as that of pathogens (including viruses) in general, is not adequately indicated by the coliform count.[52,65,101,103,131,184,185] For example, *Aeromonas* spp. have been detected when coliforms have not been.[129] The cysts of some parasites, such as *Giardia* and *Cryptosporidium*, are more resistant than coliform organisms to disinfection. Thus, the absence of coliforms in bottled water that has only been chemically disinfected will not necessarily indicate freedom from *Giardia* cysts, amoebae, and other parasites.[11,44,185] Therefore, the use of the coliform test by itself may not be adequate and, consequently, Canada is implementing the use of other indicators (*A. hydrophila, P. aeruginosa*, and others; Table 32-4) to ensure safety.

Thermotolerant (fecal) coliforms do not reliably predict the presence or absence of enteric viruses in a sample[52,55,126,127] as several enteric viruses are more resistant than *E. coli* to adverse environmental conditions.[57] Fifty-six percent of drinking water samples from various sources in Cairo, Egypt, were found to contain coliphage, while only 3% of the same samples were positive for total coliforms and fecal coliforms[55]; bacterial viruses (bacteriophages) are similar to human enteric viruses in terms of resistance to environmental factors.[76] Male-specific RNA coliphages have been proposed as indicators of fecal contamination of water.[78,79,183] These F-pilus specific bacteriophages (FRNA phages) require the presence of the F-pilus on the bacterial host for replication. Because the F-pilus is not produced at temperatures less than 30°C, FRNA phages cannot replicate in the environment. The inability of FRNA phages to replicate in the environment, combined with the high levels of FRNA phages present in human sewage, makes FRNA phages a useful indicator of fecal contamination. The use of bacteriophages as models of human enteric viruses in the environment has been the subject of a recent review.[77]

32.6.3 *Pseudomonas aeruginosa*

P. aeruginosa standards are included in Codex guidelines for source waters and natural mineral waters, as described in the "World-wide Codex Standard for Natural Mineral Waters,"[37,177] as well as in standards stipulated by the European Economic Community (EEC).[9,10] *P. aeruginosa* is cited as an indicator organism in the proposed new Canadian regulations for all bottled water[177] for the following reasons:

1. *P. aeruginosa* has been found in bottled water from a variety of countries, including Brazil, Canada, France, Germany, Spain, and the US.[70,84,136,155,177]
2. It does not occur in pristine springs.
3. The presence of *P. aeruginosa* can indicate serious contamination by pollution because it is associated with surface run-off,[168] human fecal matter,[84,160] and domestic and agriculture effluent.[186]
4. *P. aeruginosa* is an important indicator of water quality[52] because it has been the causative agent in waterborne and foodborne disease.[84,169,182] In hospital environments, especially for immunocompromised individuals, *P. aeruginosa* can cause diarrhea and can be life-threatening,[8] therefore special precautions would be justified.[169] For infants, contaminated water supplies may be a more significant source of infection by *P. aeruginosa* than foods[169]; it has been associated with illness in infants fed formula made with bottled water (reference 7 and Dr. F.A. Rosenberg, personal communication).
5. *P. aeruginosa* can also be used as an indicator of GMP because it can often be isolated from the final product, although it may not be detected from the source water. Contamination during bottling may be the result of colonization of equipment. Rubber seals or washers, linings, or coatings and grease containing organic compounds, even disinfecting soap, may all provide nutrients for this contaminant (Health Canada[168] and unpublished data).
6. Pathogenic isolates and other strains can grow in low nutrient water, such as deionized and distilled water[70,71,84,159,179,180] and can reach levels of 10^4 cfu ml^{-1} in mineral water,[71] thus increasing the risk of disease.
7. The presence of *P. aeruginosa* may impair standard coliform enumeration procedures.[3]
8. *P. aeruginosa* contamination can affect water color, clarity, and taste.[168]
9. Many of the *Pseudomonas* spp. isolates from mineral waters are highly resistant to commonly used antibiotics.[155]
10. *P. aeruginosa* potentiates the survival of *Aeromonas*,[179] *Salmonella*, and possibly other pathogens.

32.6.4 *Escherichia coli*, Fecal Streptococci, Sulfite-Reducing Anaerobes, and Parasites

Canada, Codex Alimentarius, and the EEC have specified limits for *E. coli*, fecal streptococci, sulfite-reducing anaerobes, and parasites in bottled water (Table 32-4). Similarly, Leclerc et al.[116] have indicated that bottled water analysis should include *E. coli*, fecal streptococci, and *P. aeruginosa*. As guidelines, the limits for these microorganisms can be used at the water source and at critical control points in the plant to determine the quality of the water and whether the plant is operating under GMP.

These microorganisms are useful indicators of fecal contamination.[29,110,168,186] Recovery of these organisms from any water source may mean that fecal material has gained access to the water, rendering it unfit for human consumption.[168] It is recommended that a variety of indicator organisms be included in routine testing to adequately ensure the safety of bottled water.

The detection of coliforms in the absence of *E. coli* can mean that a water source is contaminated with surface water or waters draining from surface soils. If fecal streptococci are recovered with coliforms, this suggests that the coliform contaminants have a fecal origin.[168] Thus, enterococci can be used to assess the significance of a doubtful coliform test, particularly if coliforms are found in the absence of fecal coliforms[184,185] or to confirm fecal contamination.

Zmirou et al.[192] claim that fecal streptococci are the best indicators of the risk of developing acute gastrointestinal disease when ingesting bacteriologically substandard drinking water. In general, fecal streptococci survive longer in well water than do coliforms and most enteric pathogens.[129] In a study of the occurrence of viruses in drinking water, enteric viruses or rotaviruses were detected in four samples that met acceptable levels of turbidity and residual chlorine.[105] None of the samples containing viruses had detectable total coliform bacteria; however, all samples containing viruses also contained fecal streptococci. Given their relatively high resistance to unfavorable external conditions, fecal streptococci are useful index organisms for viral contamination of water.[105,133] They are easily detected in water because of characteristics that readily separate them from other groups of bacteria.[56] Absence of fecal streptococci could indicate a safe water supply.

The spores of *Clostridium* spp. can survive longer in water than coliforms, *E. coli*, and other bacterial indicators, and may be used to indicate that contamination occurred some time ago.[168,184–186] Thus, one of the more attractive features of using clostridia as an indicator is that there is less concern about the time required to return the sample to the laboratory for assay.[26] Also, contamination of bottled water with clostridia could be indicative of ground water contamination or adulteration of the well or source with surface water,[160] or poor GMP.

Protozoan parasites such as *Giardia* and *Cryptosporidium* have caused recent waterborne disease outbreaks.[44,69,87,88,91,98,128] Such outbreaks, which include some due to municipal water, were the result of the contamination of surface water. However, 11% of the outbreaks between 1965–1984 in the US were caused by contaminated, untreated, and inadequately treated springs and wells.[44] A minimal treatment for source waters must include filtration for cyst removal, in series with disinfection for pathogenic bacteria and viruses.[63]

Although most manufacturers of bottled waters use "approved source" water that includes protected underground and further processed municipal supplies, some bottlers may use other sources. The source waters may include mountain streams, glacial waters, rivers, lakes, and ground waters, which have the potential for contamination with parasites. Several types of protozoa can be present in ground water and in very permeable aquifer layers[68] and bottled water in Mexico and France has been found to contain parasites.[47,151] *Cryptosporidium parvum* is transmitted by ingestion of oocysts spread by fecal contamination. *Cryptosporidium* oocysts are common in the environment and have been found in 65% to 97% of surface waters tested throughout the US.[114,153,154] A massive waterborne outbreak of watery diarrhea occurred in Milwaukee in 1993, which affected an estimated 403,000 individuals.[124] This outbreak was caused by *Cryptosporidium* oocysts that passed through the filtration system of a municipal water treatment plant. The likelihood of bottled water containing *Cryptosporidium* oocysts depends on the source.[11,124] In a study of an outbreak of cryptosporidiosis, Goldstein et al.[69] stated that "the use of bottled water was protective against this infection, but only if it were the sole source of drinking water." Bottled water derived from protected well or underground spring water sources is more likely to be free of *Cryptosporidium* oocysts than surface water. Water treatment before bottling, by reverse-osmosis filtration or distillation, will ensure removal of oocysts.[11] Water that has been passed through an "Absolute" 1 µm filter, but not a "Nominal" 1 µm filter, should not contain oocysts.[11]

32.6.5 *Aeromonas hydrophila*

Aeromonas hydrophila (Chapter 38) has been cited in the proposed new Canadian regulations as an indicator organism for the following reasons:

1. Bottled water from Canada, Saudi Arabia, Spain, and other countries[70,163,176] has been shown to contain *A. hydrophila*.
2. Although *Aeromonas* spp. have a poor correlation with survival of most other pathogens in water,[129] they can be discharged with, and isolated from, fecal matter,[167] may be considered as indicators of such pollution, and may have a correlation with fecal indicators in some waters.[14]
3. *A. hydrophila* has been implicated as a foodborne pathogen by an abundance of indirect evidence, is now considered by some workers a primary infectious agent,[167] and to cause waterborne disease.[26,109]
4. The presence of *A. hydrophila* (and other species) is indicative of inadequate good manufacturing practices. It seems prudent to recommend that individuals involved in the manufacturing, distribution, and preparation of foods, including water, consider *A. hydrophila* and other pathogenic *Aeromonas* spp. as undesirable in their products.
5. High levels of these bacteria may render these products unsafe. Pathogenic isolates of *A.*

hydrophila have been found to survive and proliferate to levels $>10^5$ cfu ml^{-1} in bottled water stored at room temperature.[179]

6. *A. hydrophila* has long been recognized as an opportunistic pathogen in hosts with impaired immune function.[167] Active steps may be needed to limit exposure of these populations to these potential enteric pathogens.[23]

32.7 CONCLUDING REMARKS

As with a great number of other foods, better education and communication between the bottled water associations, governments, health agencies, the media, the public (including susceptible populations), and retail outlets is badly needed. This should include the following:

1. **The types of bottled water and bacterial content.** Information on the differentiation between "natural" waters and other bottled waters is greatly needed. This information should include statements indicating that bottled water (especially "natural" waters) is not a sterile product and contains a variety of innocuous bacteria. Explanations about production under GMP, including statements about testing for pathogens and indicator bacteria would also be helpful.
2. **The handling of products.** Most persons assume that bottled water is sterile and fail to comprehend that bottled water can support a bacterial population. Having a natural bacterial content and being able to support contaminants should affect the manner in which the consumer (and especially susceptible persons) handle the product upon purchase. This should include refrigeration, proper preparation of infant formulas and other foods, as well as regular cleaning of water coolers and dispensers. Retail outlets must be encouraged to rotate their bottled water stocks on a regular basis so the consumer is purchasing the freshest product possible.
3. **Letters and communiques.** Bottled water associations, government, and health agencies should have information letters or communiques available on bottled waters to parallel those available for the safe use and handling of other foods.
4. **Labeling.** Labeling of finished products with the bottled water associations' logos or with a statement referring to membership in these trade associations would enable the consumer to purchase products produced under their strict and sanitary codes of practices.

Implementation of new standards and guidelines, as suggested in Table 32-4, is necessary to aid government agencies, food testing laboratories, and bottled water manufacturers in providing the public with the best product possible. It is not surprising that both bottled water and municipal waters contain the same microorganisms (Table 32-1) because both can originate from the same sources. These bacterial genera are found in products from a variety of countries, and many are a cause for concern because of their potential pathogenicity. When one considers that the target consumer populations may include pregnant women, the infirm, the immunocompromised, the elderly, and other susceptible people, bottled water must be shown to be safe. However, bottled water of excellent quality can be produced by following GMP/HACCP programs and by testing the source waters, in-line samples, and finished products for a variety of indicator organisms (Table 32-4) while using ACC as an indicator of GMP within 24 hours of production. New standards should include mandatory sterilization or disinfection of final products when bottled water is not manufactured from "approved sources." This would also include a mandatory filtration step or system to entrap parasites and their cysts. The definition of approved source needs to be well defined and even stated in regulations. Bacteriological analysis of approved sources must include all the indicator microorganisms listed in Table 32-4.

More extensive surveillance of the bottled water industry is recommended, especially of those manufacturers who are not members of the bottled water associations (eg, IBWA and CBWA). Public health officials should be encouraged to inquire about ingestion of bottled water when investigating waterborne and foodborne illness. More surveillance by government laboratories is often impractical because of limited resources. However, Geldreich[6] recommended a two-tiered monitoring approach whereby industry would conduct 90% of the monitoring, using certified labs, while government agencies would conduct the remaining 10% to verify data. This self-governing approach is consistent with both the aims of the IBWA and CBWA, which serve as self-monitoring trade associations.[187] The standards established by these organizations for the production of bottled water require that all plants be inspected on an annual basis by an independent agency in addition to government inspections. These organizations welcome additional and more stringent regulations.[187] However, not all bottled water manufacturers belong to these organizations and thus do not comply with their codes of practice.[95]

Each container should be marked with the bottling date, date of expiration, and lot number to assist the retailer and consumer in determining freshness.[66,156] These will aid in the follow-up of consumer complaints and the removal of product from retail shelves. Each container should be supplied with a protective seal to reduce the chance of contamination[6] and tampering.

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33 Spices and Herbs

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33.1 INTRODUCTION: DEFINITIONS AND IMPORTANCE

According to the International Trade Centre UNCTAD/GATT, "spices may be defined as one of the various strongly flavored or aromatic substances of vegetable origin obtained from tropical and other plants, commonly used as condiments or employed for other purposes on account of their fragrance and preservative qualities."[3] Condiments are spices alone, or blends of spices that have been formulated with other flavor potentiators to enhance the flavor of foods. The so-called "true spices" (eg, pepper, cinnamon, nutmeg, and cloves) are products of tropical plants, and may be fruit, bark, buds, roots, or other parts. Herbs are usually from leafy parts of soft-stemmed plants of the temperate zone (eg, oregano, marjoram, basil). Spice seeds (eg, mustard, caraway, celery, anise) may be either from tropical or temperate areas. Dehydrated vegetable seasonings (eg, onion or garlic powders) are flavorings with similar uses and problems to those of spices and herbs.

Spices, herbs, and dry vegetable seasonings are chemically and histologically a very heterogeneous group of commodities. Their important properties, processing, and uses are reviewed in several monographs.[49,57,122]

Spices and herbs are important items in international trade. In addition to their household uses, they are used in virtually all categories of the food industry. The markets for spices and herbs in industrialized countries are increasing with the growing popularity of ethnic foods.

33.2 RELATIONS OF SPICES AND HERBS TO MICROBIOLOGICAL QUALITY AND SAFETY OF FOODS

The food microbiologists' interest in spices and herbs is based principally in the facts that they may (1) exhibit antimicrobial activity, (2) occasionally stimulate microbial metabolism, (3) become moldy if held at improper humidity, and (4) often contain large numbers of microorganisms that may cause spoilage or, more rarely, disease, when introduced into food.

33.3 SPICE ESSENTIAL OILS AND OLEORESINS

Spice essential oils are volatile aromatic substances obtained from ground spices by steam distillation, while oleoresins are prepared by extraction of spices using suitable organic solvents or liquid/supercritical CO_2.[33,102] These products offer certain advantages over natural spice powders, ie, freedom from enzymes and microorganisms, uniform dispersion in products, and easy handling and storage. They may be mixed with a carrier (eg, salt) or encapsulated (eg, in starch). However, for certain products they are unable to substitute all functional properties of the original spices, particularly the aroma profile or appearance of the product. For example, in products such as certain sausages in which the spices are visible, particularly pepper and cloves, this is a part of the distinctive quality of the particular meat product. Also the loss of flavor of spice extracts during any cooking process, unless fully enclosed, is greater than that of natural spice. Encapsulated spices retain volatiles better than extracts.[133] The production of a new spice alternative designated "Mildspice-Registered" involves mixing natural spice particles and spice extractives and microencapsulation of this mixture in a dry matrix.[37] Micro-Registered is claimed to combine the true flavor profile of traditionally ground spices with the low microbial counts and convenience of encapsulated variants.

33.4 ANTIMICROBIAL EFFECTS AND ANTIMICROBIAL CONSTITUENTS

There is considerable interest in the use of naturally occurring compounds as alternative food additives to prevent or delay the growth of foodborne microorganisms.[14] Among these materials, the antimicrobial activities of extracts of several spices and herbs have been recognized for many years. Various essential oils and/or their components have been reported as being active against a wide range of bacteria (including spore-formers), and fungi (including yeasts). The major antimicrobials present in some spices and herbs are listed in Table 33-1.

Table 33-1 Major Antimicrobials Present in Some Spices

Antimicrobials	*Spices*	*References*
Allicin	Garlic	138
Allyl isothiocyanate	Mustard	32
Anethol	Anise	51, 140
Capsicidine	Hot paprika	55, 56
Capsaicine	Cayenne, hot paprika	55, 56
Carnosol	Rosemary	21
Carvacrol	Oregano, savory, thyme	11, 80, 84
Cinnamic aldehyde	Cassia, cinnamon bark	51, 116
Cuminaldehyde	Cinnamon, cumin seed	84
Eugenol	Allspice, cloves	17, 51, 96
Geraniol	Ginger, thyme	80
p-menthane compounds	Mint	143
Thymol	Oregano, thyme	11, 84
Ursolic acid	Rosemary	21

The antimicrobial activity of a given spice and extract from it may depend on only one or two of the large number of different chemical compounds that make up the spice oil. Some of the antimicrobial molecules may be common to several spices, whereas others are unique to a few closely related spices. However, the composition and content of essential oils varies from spice to spice and even within the same spice, depending on agricultural practices, geography, and climatic conditions during the growing season. The early literature of the antimicrobial effects of herbs, spices, and their extracts has been reviewed extensively.[36,64,111]

Spices and herbs that contain the most inhibitory essential oils are cloves, thyme, oregano, cinnamon, allspice, cumin, and caraway.[12,69] Onion and garlic juice also contain antimicrobial compounds.[1,53] In cloves and allspice, the major antimicrobial components are eugenol, eugenol acetate, or eugenol methylether; in cinnamon and cassia, they are cinnamic aldehyde, cinnamic acetate, and eugenol; in thyme, they are thymol and carvacrol; in mustard seed, it is allyl isothiocyanate; and in garlic, it is allylsulfonyl allyl sulfide.[9,42,69,71,72,78] The degree of observed microbial inhibition by spices depends on their concentration in media, the method employed to test for antimicrobial activity, and the strains/species of microorganisms.[7,69] There is some evidence that synergistic or cumulative inhibitory effects exist between the components in certain spice extracts.[17,115]

The concentrations of essential oils in spiced food are generally too low to prevent microbial growth.[127,160] Furthermore, levels found to be inhibitory in laboratory media are often insufficient to cause inhibition in foods.[41,69] On the other hand, the antimicrobial potential of essential oils may be enhanced in food in which the contaminants have been stressed.[22,23] In some cases the preservative effect of essential oils may be of practical importance.[17] Cinnamon (about 1%) in raisin bread decreased mold growth and even smaller concentrations prevented production of aflatoxin.[16] In recent studies in Germany, the addition of 11 different spices was tested for controlling mold growth on sliced toast bread. Cloves and oregano in concentrations of 1% to 2% significantly delayed growth of various bread-relevant fungi.[90] Further studies with proper spice mixtures are required to optimize sensory quality and antifungal efficiency for bakery products.

The efficacy of oregano essential oil and to a lesser degree of thyme essential oil as fumigants against fungi (*Aspergillus flavus, A. niger,* and *A. ochraceus* as well as natural microflora of wheat grain) was demonstrated by Paster and coworkers.[115] The minimal inhibitory concentration of oregano oil applied for 24 hours as fumigants was 2.0 μl L^{-1} and it exhibited fungicidal activity at 20 μl L^{-1} level. Because the essential oils tested caused a reduction in the germinability of grains, these materials cannot be used for seed preservation but only to preserve grains destined for consumption.

Addition of 0.5% fresh, finely ground rosemary or 1% rosemary oil to ready-to-eat pork liver sausage before cooking delayed listerial growth during refrigerated storage, however, these levels imparted strong odor and flavor to the product.[114] The antilisterial activity of rosemary appears to be associated with its antioxidant compounds. An antioxidant extract of rosemary (STABEX) obtained by liquid carbon dioxide extraction was an effective antilisterial additive at 0.5% in the same meat product without affecting its characteristics.

The mechanisms of inhibition and destruction of microorganisms by essential oils are varied because their antimicrobial compounds are chemically diverse. It is suggested, because of the similarity in structure of the active components of some essential oils with phenols, that spice oils may affect growth by the same mechanism as phenols. Phenols affect the cell membrane of bacteria by altering permeability and interfering with transport systems, electron transport, and energy production.[71,155]

33.5 STIMULATION OF MICROBIAL ACTIVITIES

It has been observed that some spices accelerated the production of lactic acid and the decrease of pH during production of fermented sausages in which lactic acid bacteria were used as starters.[48,83,161] The stimulatory factor seems to be the manganese content of such spices, a known essential element in the metabolism of lactic acid bacteria.[48,107]

33.6 MICROBIOLOGICAL CONTAMINATION OF SPICES AND HERBS

33.6.1 Sources of Microbial Contamination

Spices and herbs contain those microorganisms indigenous to the soil and plants where they are grown and that survive the drying process. Dust, insects, fecal material from birds and rodents, and possibly the water used in some processes (eg, soaking peppercorns in the production of white pepper), are the main sources of additional contamination.

Microbial counts can vary from one storage period to another depending on moisture content and storage conditions. The viable count is a function of original bioload, proliferation, and die-off. Therefore, the observed counts of samples of the same spice or herb of various lots often show deviations of several logs.[65,69,99,113] The microbiological quality of spices and herbs often reflects the hygienic situation of the region of their production and handling.

33.6.2 Saprophytic Bacteria

The range of microbial contamination as a percentage distribution of aerobic plate counts and mold counts of various spices is charted in Table 33-2. According to this frequency distribution, the highest levels of bacterial contamination was found usually in black pepper, turmeric, the capsicum spices, coriander, and allspice,[10,27,69,85] not infrequently with mesophilic aerobic bacterial counts of $>10^7$ cfu g^{-1}.

In many spices, the aerobic mesophilic spore-forming bacteria originating from soil are dominating in the microflora.[85] Most frequently found species are *Bacillus subtilis, B. licheniformis, B. megaterium, B. pumilus, B. brevis, B. polymyxa,* and *B. cereus.*[10,50,59,74,135] Because during drying and storage many vegetative cells die off, whereas spores survive, aerobic bacterial spores may comprise more than 50% of the mesophilic aerobic plate counts (Table 33-3).[69,106] The proportion of obligately anaerobic spore-formers is usually small.[50,87,94]

Thermophilic anaerobic and aerobic spore-formers are found occasionally.[87] Thus, some spices are potential sources of very heat-resistant spores of bacteria, including thermoduric flat sours, putrefactive anaerobes, and sulfide stinkers. Psychrotrophic bacteria are generally less numerous than mesophiles in spices and herbs.[27]

Table 33-2 Percentage Distribution of Aerobic Plate Counts and Mold Counts of Various Spices

		Percentage Distribution of Aerobic Plate Counts								*Percentage Distribution of Mold Counts*							
Spice	*No. of Samples*	*<1.0 E2*	*1.0 E2[a] to 9.9 E2*	*1.0 E3 to 9.9 E3*	*1.0 E4 to 9.9 E4*	*1.0 E5 to 9.9 E5*	*1.0 E6 to 9.9 E6*	*≥1 E7*	*No. of samples*	*<1.0 E2*	*1.0 E2 to 9.9 E2*	*1.0 E3 to 9.9 E3*	*1.0 E4 to 9.9 E4*	*1.0 E5 to 9.9 E5*	*1.0 E6 to 9.9 E6*	*≥1 E7*	*Ref.*
Allspice/	33			3	7	46	42	3	27	37	22	15	18	7			69
pimento	8					25	75		8	13	37	37	13				113
Anise	22			23	36	36	5		16	56	25	6	13				69
Basil	21				14	48	38		17	65	24	6	6				69
Bay	41	5	5	46	34	7	3		35	34	29	26	11				69
Capsicum (chili)	57		2	9	28	18	31	12	59	44	15	22	7	7	5		69
	21		5	19	38	38			21	53	19	14	0	14			83
Caraway	17		12	35	29	18	6		14	57	7	29	7				69
Cardamom	15	7	13	13	27	7	33		15	67	13	20					69
Cassia	36	6	66	11	14	3			20	55	5	25	5	10			69
Chives	11	9	18	55	18				11	73	27						69
Cinnamon	42	2	5	19	48	21	2	2	51	18	33	43	6				69
	36	3	6	22	33	11	17	8	36	39	19	8	11	14	3	6	113
Cloves	28	32	21	25	18	4			26	88		8	4				69
	23	9	48	43					23	96	4						113
Coriander	30	9	3	3	30	37	13	13	23	4		61	26	9			69
	11		0	9	19	27	9	27	11	9	9	9	9	18	46		113
Cumin	12				33	42	25		8	25		62		13			69
	15		6	0	40	47	7		15	40	20	20	14	6			113
Fenugreek	10			20	30	30	20		8	13	37	25		25			69
	14		7	21	58	7	7		14	21	36	29	7	7			113
Garlic	32			16	47	28	9		15	60	40						69
	21			29	33	33	5		21	57	38	5					113
Ginger	33	3	9	21	15	45	7		28	57	14	18	11				69
	35				14	86			35	26	51	14	9				113
Mace	28			43	50	7			22	59	14	23	4				69
	22			18	18	45	14	5	22	41	13	5	9	27	5		113
Marjoram	21		5	0	19	43	28	5	14		7	64	29				69
Mint	3					33	67		3	33	67						113
Mustard seed	67	9	30	33	18	9	1		63	86	6	6	2				69
	4		25			75			4	25	0	50	25				113
Nutmeg	45	11	20	44	16	4	4		33	52	27	12	9				69
	33		21	58	18	3			33	64	27	6	3				113
Onion powder	13		15	31	54				13	8	38	54					113

continues

Table 33-2 Continued

		Percentage Distribution of Aerobic Plate Counts								*Percentage Distribution of Mold Counts*							
Spice	*No. of Samples*	*<1.0 E2*	*1.0 E2[a] to 9.9 E2*	*1.0 E3 to 9.9 E3*	*1.0 E4 to 9.9 E4*	*1.0 E5 to 9.9 E5*	*1.0 E6 to 9.9 E6*	*≥1 E7*	*No. of samples*	*<1.0 E2*	*1.0 E2 to 9.9 E2*	*1.0 E3 to 9.9 E3*	*1.0 E4 to 9.9 E4*	*1.0 E5 to 9.9 E5*	*1.0 E6 to 9.9 E6*	*≥1 E7*	*Ref.*
Oregano	56	2	4	21	41	23	9		48	35	25	33	9				69
	7		14	29	14	29	14		7	29	14	0	57				113
Paprika	80				11	9	62	18	61	44	44	7	5				69
	32	6	6	6	12	41	16	13	32	12	13	50	25				113
Parsley flakes	6			83	0	0	17		6	67	17	0	16				113
Pepper (black)	108			1	3	5	50	42	82	32	10	28	5	2	23		69
	97					10	41	49	97	32	9	5	22	29	1	2	113
Pepper (white)	42			12	26	57	5		44	2	2	34	36	23	2		69
	66				10	32	52	6	66	15	20	6	6	36	15	2	113
Sage	17			11	41	41	6		14	7	21	21	50				69
Savory	10			40	50	10			6		67	33					69
Thyme	19			5	11	32	53		16	6	0	6	81	6			69
Turmeric	24			4	0	21	46	29	32	87	3	6	3				69
	22		4	23	23	23	14	14	22	64	27	0	9				113

[a] E2 = 10 exponential 2 = 10^2.

Table 33-3 Mesophilic Aerobic Bacterial Counts and Spore Counts in Various Spice Samples

	Log cfu g^{-1} at 30°C	
Spices	*Total Aerobic Viable Count*	*Spore Count*[a]
Allspice	5.8	5.9
Caraway seed	5.2	3.4
Chili	6.0	5.8
Coriander I	6.4	5.9
Coriander II	6.0	4.5
Ginger	8.4	7.9
Marjoram	6.5	4.8
Mustard	5.8	5.7
Nutmeg	5.7	5.7
Paprika I	7.0	7.1
Paprika II	6.0	5.7
Paprika III	5.4	5.4
Paprika IV	5.0	4.5
Paprika V	4.8	4.3
Pepper, black I	8.0	8.1
Pepper, black II	7.5	7.4
Pepper, black III	7.4	7.4
Pepper, white I	5.6	4.1
Pepper, white II	5.6	5.2
Pepper, white III	3.5	3.5
Mixed spices	6.3	6.2

[a] After a heat shock at 80°C for 10 minutes.

Source: Adapted with permission from L. Neumayr, S. Promeuschel, I. Arnold, and L. Leistner, Gewürzentkeimung, Verfahren und Notwendigkeit, *Abschlussbericht fur die Adalbert-Raps-Stiftung zum Forschungsvorhaben,* © Bundesanstalt fuer Fleischforschung, Kulmbach.

A wide variety of nonsporing bacteria may also be present in spices. Coliforms are often found[113] but *Escherichia coli* is not frequent (Table 33-4).[10,113,132] Fecal streptococci occur usually in low numbers, rarely reaching the level of 10^4 cfu g^{-1}.[10] Staphylococci and lactic acid bacteria are not frequent in spices.[69]

33.6.3 Pathogenic Bacteria

Spore-forming bacteria (*B. cereus* and *Clostridium perfringens*) that are capable of causing gastroenteritis when ingested in large numbers are frequently found in spices, but usually in small numbers (Table 33-5).[26,27,86,87,113,123,126] However, in extreme cases *B. cereus* counts up to 10^5 cfu g^{-1} were found.[10,85,113,147] *B. subtilis* and *B. licheniformis,* common in many spices,[59] were occasionally responsible for foodborne gastroenteritis.[88,89] Because spores of the above organisms may survive cooking temperatures and may grow at permissive temperatures in many foods, spices harboring these spores must be considered potential health hazards.[120,121]

Although infrequently, salmonellae have been found in a variety of spices.[13,15,113,126,129] Their presence is of special concern when spices are used on foods that are consumed raw or when they are added to foods after cooking.[15] Black and white pepper have both been implicated as causes of salmonellosis due to *Salmonella weltevreden* and *S. oranienburg* infections.[61,137] In 1993, a nationwide outbreak of salmonellosis occurred in Germany because of paprika and paprika-powdered potato chips contaminated by a great number of strains.[92]

Staphylococcus aureus is rarely found in dry spices.[15,120] *Listeria* spp. seem to be even more infrequent in spices and herbs.[85]

Concerning microorganisms of public health concern at low populations in spices, it should be considered, however, that lack of resuscitation, and inhibition by spice components in the lower dilution levels, may yield incorrect results.[113,157]

33.6.4 Fungal Contamination and Mycotoxins

Aspergillus spp., *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., *Rhizopus* spp., and *Mucor* spp. are the most common molds detected in spices, and viable contamination levels of mold are in the 10^3–10^5 propagules g^{-1} range of various samples (Table 33-2).[69,99,113]

A relatively high incidence of toxigenic molds, including *Aspergillus flavus, A. parasiticus, A. fumigatus, A. ochraceus, Penicillium citrinum,* and *P. islandicum* has been detected in some spices.[2,69] Several investigators have found aflatoxins in a range of spices, although the levels were generally low.[54,69,95,99,101,102,125,158] There is no correlation between the severity of microbial contamination and the aflatoxin concentration of spices.[93] Yeasts have been found in spices in low numbers only.[10,97]

33.7 EFFECT OF PRIMARY PROCESSING, PACKAGING, AND STORAGE ON THE MICROFLORA OF SPICES AND HERBS

The various spices require widely diverse harvest and postharvest methods, which affect the microbial content in different ways.[69] The main consideration is to dry the products as rapidly as possible to prevent spoilage while retaining their desirable characteristics. Drying of spices reduces the number of vegetative bacteria present. The remaining flora consists mainly of spore-forming bacteria and

Table 33-4 Percentage Distribution of Coliform and *Escherichia coli* Counts of Various Spices

		Coliforms g⁻¹					E. coli *g⁻¹*				
Spice	*No. of Samples*	*<3*	*3–9*	*10 to 1.0 E2*[a]	*1.1 E2 to 1.0 E3*	*≥1.1 E3*	*<3*	*3–9*	*10 to 1.0 E2*	*1.1 E2 to 1.0 E3*	*≥1.1 E3*
Allspice/pimento	8	75	0	13	12		100				
Capsicum (chili)	21	81	19				95	5			
Chives	11	100					100				
Cinnamon	36	64	6	17	8	5	92	8			
Cloves	23	100					100				
Coriander seed	11	64	18	18			100				
Cumin seed	15	74	6	6	6	6	100				
Fenugreek seed	14	86	7	7			79	21			
Garlic	21	81	10	9			95	0	5		
Ginger	35	60	20	14	6		91	0	9		
Mace	22	63	5	18	9	5	82	14	4		
Mint flakes	3	33	0	0	67		67	33			
Mustard seed	4	25	25	50			100				
Nutmeg	33	91	9				100				
Onion powder	13	31	31	38			92	0	8		
Oregano	7	43	29	28			100				
Paprika	32	28	13	56	0	3	97	3			
Parsley flakes	6	83	0	7			100				
Pepper, black	97	31	10	11	11	37	67	8	13	7	5
Pepper, white	66	15	8	32	21	24	67	13	15	5	
Turmeric	22	91	0	5	0	4	95	0	5		

[a] E2 = 10 exponential 2 = 10^2.

Source: Adapted with permission from J. Pafumi, Assessment of the Microbiological Quality of Spices and Herbs, *Journal of Food Protection*, Vol. 49, pp. 958–963, © 1986, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

molds, because of their ability to survive long periods on dried materials. The number of viable microorganisms present is a function of packaging and storage conditions. Because of slow oxidation, viable cell counts of dry spices and herbs may decrease during storage. This may partly be the reason why spices and herbs are, in general, apparently more heavily contaminated at import than at retail.[77,132] In a Finnish study, Malmsten et al.[94] found greater viable cell counts in freeze-dried and vacuum-packaged herbs than in those stored in oxygen-containing packages (glass jars) (Table 33-6), and microbial counts were generally higher in samples stored at 23°C than at 35°C. Survival of molds and yeasts inside packages was especially dependent on the presence of oxygen, whereas the dried herbs in different types of packaging contained similar numbers of viable aerobic sporeformers. Of course, the storage conditions that maintain higher viable counts also maintain better color and other sensory qualities.[117,118]

33.8 APPLICATION OF GOOD HYGIENIC PRACTICE AND THE HAZARD ANALYSIS CRITICAL CONTROL POINT CONCEPT

A proposed Draft Code of Hygienic Practice for Spices and Dried Aromatic Plants is under preparation by the Codex Committee on Food Hygiene,[20] describing requirements for environmental hygiene in the production and harvesting area, establishment design, and facilities; personnel hygiene and health requirements; hygienic processing requirements; and end-product specifications. Application of the Hazard Analysis Critical Control Point (HACCP) system in spice production is illustrated in general terms in the example of black pepper by the International Commission on Microbiological Specifications for Foods.[70] Similarly, the American Spices Trade Association published a "Spice Microbiology Hazard Background Review" to lay the groundwork for applying HACCP concepts to the spice trade.[6]

Table 33-5 Distribution Range of *Bacillus cereus* in Various Spices

		Percentage Distribution of Bacillus cereus Counts g^{-1} [a]				
Spice	*No. of Samples*	*<1.0 E2[b]*	*1.0 E2 to 9.9 E2*	*1.0 E3 to 9.9 E3*	*1.0 E4 to 9.9 E4*	*1.0 E5 to 9.9 E5*
Allspice/pimento	8	25	38	37		
Capsicum (chili)	21	70	10	10	10	
Chives	11	100				
Cinnamon	36	42	14	39	5	
Cloves	23	100				
Coriander seed	11	82	18			
Cumin seed	15	13	27	33	20	7
Fenugreek seed	14	57	15	21	7	
Garlic	21	67	23	10		
Ginger	35	20	23	54	3	
Mace	22	18	14	64	4	
Mint flakes	3	0	0	100		
Mustard seed	4	50	50			
Nutmeg	33	76	21	3		
Onion powder	13	77	15	0	8	
Oregano	7	57	0	14	29	
Paprika	32	62	19	19		
Parsley flakes	6	83	17			
Pepper, black	97	19	8	56	16	1
Pepper, white	66	48	17	33	2	
Turmeric	22	50	18	22	5	5

[a] Detection limit: 10^2 g^{-1}.

[b] E2 = 10 exponential 2 = 10^2.

Source: Adapted with permission from J. Pafumi, Assessment of the Microbiological Quality of Spices and Herbs, *Journal of Food Protection*, Vol. 49, pp. 958–963, © 1986, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

Steps in the production of black pepper (probably the most important commodity of the international spice trade) and typical critical control points (CCPs) are shown in Figure 33-1.[70]

Black pepper is produced from the unripe dried berry of a perennial climbing vine (*Piper nigrum* L.) of far eastern tropics.[49] While the time of harvesting is primarily determined by the maturing of the berry, pepper should not be harvested in wet weather unless mechanical means of drying are available. The moisture content of harvested berries must be reduced to 10% to 15% to minimize microbial growth.

Table 33-6 Log$_{10}$ Microbial Counts of Freeze-Dried Herbs after Storage at 23°C (results of counts on five replicates)

Herb	*Microbial Group*	*Glass Jar*	*1 Year Vacuum*	*Nitrogen*	*Glass Jar*	*2 Years Vacuum*	*Nitrogen*
Dill	APC[a]	4.9	5.3	4.3	4.6	5.3	4.9
	M&Y[b]	3.7	4.0	3.0	2.2	2.6	3.0
Basil	APC	4.7	4.9	5.0	4.5	5.6	5.5
	M&Y	3.0	3.5	3.6	2.7	4.0	3.8
Marjoram	APC	5.8	6.3	5.8	5.5	6.5	6.6
	M&Y	2.3	2.3	2.9	1.8	2.7	1.5
Wild marjoram	APC	5.3	5.8	5.8	4.8	6.0	4.9
	M&Y	2.0	3.6	2.7	1.2	2.7	2.2

[a] APC = aerobic plate counts.

[b] M&Y= molds and yeasts.

Source: Reprinted with permission from T. Malmsten, K. Paeaekkoenen, and L. Hyvoenen, Packaging and Storage Effects on Microbiological Quality of Dried Herbs, *Journal of Food Science*, Vol. b56, pp. 873–875, © 1991, Institute of Food Technologists.

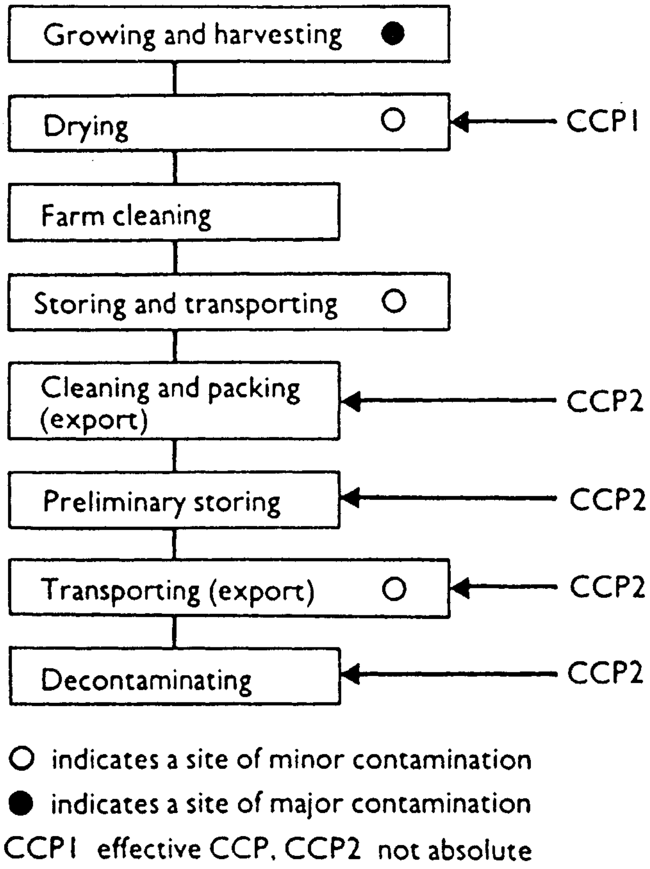
Growing and harvesting ●
Drying ○
CCP1
Farm cleaning
Storing and transporting ○
Cleaning and packing (export)
CCP2
Preliminary storing
CCP2
Transporting (export) ○
CCP2
Decontaminating
CCP2
○ indicates a site of minor contamination
● indicates a site of major contamination
CCP1 effective CCP, CCP2 not absolute

Figure 33-1 Flow Diagram for Black Pepper Production. *Source:* Reprinted with permission from ICMSF (The International Commission on Microbiological Specifications for Foods), Production and Harvesting of Plant Foods, in *Microorganisms in Foods, 4, Application of the Hazard Analysis Critical Control Point (HACCP) System to Ensure Microbiological Safety and Quality,* pp. 139–175, © 1988, Blackwell Science, Ltd.

The bulk of the world's pepper production is sun dried. Mechanical drying results in lower microbial contamination. For solar drying the provision of protection from rain, dew, and vermin, and for mechanical drying the cleanliness of the drier and time and temperature of the drying process, are important factors of control. The moisture of the dried berries may be determined. After farm cleaning and preliminary storing of dried peppercorn, pepper is transported to export centers. During storage and transport, it should be protected from insects, high humidity, and rain. Redrying (if necessary), final cleaning, eventually fumigation (for disinfestation), grading, and packing are usually done at exporting centers. At bagging, the moisture content of pepper should be 11% or less. Awaiting and during export, pepper should be stored in moisture-proof bags or at a relative humidity not above 70%. Protection from moisture, prevention of large temperature changes that may create moisture migration and localized moist areas, and freedom from vermin are important control requirements during storing and transport to importing countries. Microbiological decontamination procedures, dealt with in later parts of this chapter, have their specific CCPs appropriate to the specific technologies.

33.9 MICROBIAL SPOILAGE OF SPICES AND FOODS BY MICROORGANISMS FROM SPICES

Because of their low water activities (normally within the range of 0.3–0.65), there is virtually no bacterial spoilage of spices and herbs after drying. However, fungi may grow on them prior to drying and during drying, storing, and shipping, if relative humidity and temperature are permissive, or localized wetting occurs.[74,134] Insect infestation may contribute to the biodeterioration of spices, by carrying mold spores.[136]

Bacterial spore populations in ingredients to be used in low acid products that undergo a mild heat treatment, eg, canned pasteurized luncheon meats, may cause spoilage if mishandling occurs in marketing channels or by consumers. For the canning industry, if an ingredient contributes a heat-resistant microflora in terms of the process being applied, ie, thermophilic spores, then that ingredient becomes the most important determinant of the bacteriological quality of the product, and control over the finished product requires effective control over the spore load of the ingredient.[6,142] Spices can play a major etiological role in mold contamination of certain food products[63,75] and they can cause spoilage if the products provide a proper environment for growth of the molds, and they do not undergo processing that would destroy the fungi. Thus, it is important to determine the types of organisms that could spoil a product and set specifications (microbial or process) for the particular organism or group of organism.[6]

33.10 METHODS OF MICROBIOLOGICAL DECONTAMINATION

In a number of products, the introduction of spoilage organisms or pathogens from spices and herbs must be controlled carefully.[57] In such instances, the use of essential oils and oleoresins or spices that have been treated to reduce the levels of microorganisms is warranted or advisable. Even where there is a low probability of contaminated ingredients causing spoilage, ingredients may introduce numbers of microorganisms considered undesirable to industrial and regulatory interests.[62] However, spices should not be accepted for processing treatments if they show signs of insect damage or mold growth because of the danger that they may contain mycotoxins.[20] Once treated or otherwise proven to be pathogen-free, spices, like any other food product, must be handled to avoid recontamination. Manufacturing processes such as cleaning, sifting, and grinding provide ample opportunity for cross-contamination to occur, and to be minimized, eg, by application of the HACCP system.[6]

33.10.1 Gas Treatments

Until the early 1980s, the most widely used method to destroy microorganisms in dry food ingredients was fumigation, ie, treatment with ethylene oxide or to a much lesser extent, propylene oxide.[58] Because of the extreme flammability of ethylene oxide, various nonflammable mixtures of ethylene oxide in inert gases were applied. The fumigation treatments were carried out in specially designed vacuum chambers. The concentration range of ethylene oxide used for microbiological decontamination is 400 to 1,000 mg L^{-1}. Under industrial conditions, ethylene oxide treatment reduces the aerobic plate count of spices by 1 to 4 logs depending on the type of spice, the composition of the microflora, and conditions of the treatment.[44] Bacterial spores are only marginally more resistant than vegetative cells. Decrease of mold counts is usually 2 to 3 logs, occasionally more. The rate of destruction of microbial cells depends on the concentration of the fumigant, temperature, relative humidity of the atmosphere in the fumigation chamber, the moisture content of the product treated, the porosity of the product, and the permeability of the packaging material.[68,69] However, because of toxicological considerations, the use of ethylene oxide is now discouraged or even banned for food uses in most countries.[38,103,112]

Decontamination efficacies of ethylene oxide fumigation and other treatments described in subsequent pages can be compared in Tables 33-7 and 33-8.

According to Zhao and Carston,[164] ozone treatment of aqueous slurries of black peppercorns reduced the microbial population by 3–4 log without significant effects on the volatile constituents of the whole peppercorn. Sparging ground black pepper at various moisture levels with ozonized air for up to 6 hours resulted in similar reductions in the viable cell count. However, it caused oxidation of certain volatile oil constituents. Earlier German studies[159] showed also serious flavor changes of various ozone-treated spices, thus, the technological feasibility of ozone treatment for decontamination of spices is very limited.

33.10.2 Irradiation

Ultraviolet irradiation has been tried,[24,40] however, it seems to be unsuitable for commercial purposes, because its penetrating capacity is too low for effective commercial decontamination of dry ingredients.

Unlike UV irradiation, treatment of spices and herbs with Co60 gamma irradiation and machine-produced X-rays or accelerated electrons are viable and safe processes for destroying contaminating organisms in a wide range of products and packaging, with little change of sensory properties.[44,113,119,131,139] The safety and wholesomeness of irradiated foods have been more thoroughly studied than those produced by any other food processing method.[35]

Except for differences related to penetration and eventual dose-rate effects,[73] electromagnetic ionizing radiations and electron beams are equivalent in food irradiation.[152] Depending on the design of the irradiation facility and requirements, products can be treated in bulk or packed forms. Spices are best packaged before irradiation in order to avoid recontamination after treatment (see Chapter 4).[91]

A radiation dose of up to 20 kGy may be required to achieve "commercial sterility" (ie, a count of mesophilic aerobic bacteria of less than 10 cfu g^{-1}) in natural spices and herbs; however, doses of 3 to 10 kGy can reduce viable counts to a satisfactory level (from 10^5–10^7 cfu g^{-1} to less than 10^3–10^4 cfu g^{-1}, see Table 33-7) without affecting quality attributes.[39,46,73,104,105,141,144,146,154] Thermophilic spore-forming bacteria of great importance to the canning industry can be practically eliminated with the same radiation doses as those necessary to cause a sufficient reduction of the mesophilic viable counts (Table 33-9). Bacteria of the family Enterobacteriaceae are relatively radiation sensitive even in dry ingredients, and in most cases a dose of approximately 5 kGy is sufficient to reduce their numbers by a factor of at least 10^3, ie, to undetectable levels.[44] A dose of 4–5 kGy can likewise eliminate molds at least as effectively as ethylene oxide treatment (Table 33-8).

The surviving microflora of spices treated with (pasteurizing) doses of ionizing radiation have lower heat

Table 33-7 Comparison of Decontamination Efficacy of Various Treatments on the Viable Mesophilic Bacterial Counts of Some Spices

	Ethylene Oxide				*Ionizing Radiation*				*Thermal Methods*			
Spice	*Fumig. Condit.*	*Initial Count*^{a}	*Lethality*^{b}	*References*	*Rad. Dose, kGy*	*Initial Count*	*Lethality*	*References*	*Thermal Method*	*Initial Count*	*Lethality*	*References*
Allspice (pimento)	1000 g ETOX m^{-3} 6hr	6.6	5.0	98	2	6.2	1.2	153	"Sterispice" process	5.0	>3.7	145
					4		2.2					
					6		3.2					
					8		4.9					
					10		>5.2					
Caraway	250 g m^{-3} 6hr, 22°C	4.5	1.5	58	5	5.5	0.5	8	"HF treatment"	5.4	1.7	28
					7.5		2.9					
					10		3.2		"MicroControl" steam treatment	4.3	1.6	30
	750 g m^{-3} 9hr, 22°C		2.5						"EVW" steam treatment	5.5	1.5	30
Cayenne	500 g ETOX m^{-3} 6–7hr, 25°C	7.0	5.2	25	3	7.5	2.0	52				
					4.5		4.0					
					6		5.3					
					7.5		5.8					
					10		>6.5					
Cinnamon	1500 g m^{-3} 8hr, 20–25°C	4.2	>3.2	150	2	4.2	0.5	150	"HF treatment"	5.0	1.6	28
					4		0.8					
					6		>2.2					
									"EVW" steam treatment	5.2	3.2	30
Coriander	500 g ETOX m^{-3} 6–7hr, 20–25°C	6.4	3.7	25	5	6.7	3.8	162	"Sterispice" process	6.0	>4.7	145
					10		>5.7					
Fenugreek	1500 g m^{-3} 8hr, 20–25°C	4.9	>3.9	150	2	4.9	1.3	150				
					4		1.5					
					6		2.6					
Garlic powder	800 g m^{-3} 6hr, 22°C	5.9	0.6	106	4	5.9	>4.9	106				
	5hr	4.7	0.5	153	5	4.7	3.4	153				
					7.5		4.2					

continues

Table 33-7 Continued

	Ethylene Oxide				Ionizing Radiation				Thermal Methods			
Spice	Fumig. Condit.	Initial Count[a]	Lethality[b]	References	Rad. Dose, kGy	Initial Count	Lethality	References	Thermal Method	Initial Count	Lethality	References
Ginger	1500 g m^{-3} 8hr, 20–25°C	6.8	3.7	150	2	6.8	1.2	150	"Sterispice" process	6.0	>5.0	145
					4		2.7					
					6		3.2					
Mace	1000 g ETOX m^{-3} 6hr	4.4	2.6	98	5	4.6	1.9	148	"Enclosed MW system"	4.8	1.2	28
					7.5		>3.6					
Marjoram	500 g ETOX m^{-3} 6–7hr, 20–25°C	6.9	5.2	25	4	6.8	4.3	106	"Enclosed MW system"	5.7	1.9	28
					8		>5.8		"HF treatment"	6.0	1.7	28
									"Zelgerm" steam treatment	5.2	2.0	31
Nutmeg	1500 g ETOX m^{-3} 6hr	4.3	3.0	98	5	4.6	>3.6	149	"Sterispice" process	5.0	>4.0	145
Onion powder	800 g m^{-3} 6hr, 22°C	5.2	0.6	46	4	5.2	1.6	46				
					7.5		2.1					
Paprika	500 g ETOX m^{-3} 6–7hr, 20–25°C	6.8	>5.8	25	2	6.8	3.3	79	"Enclosed MW system"	9.0	1.8	28
					4		4.3			9.4	3.3	
	1000 g ETOX m^{-3} 6hr	6.2	1.9	98	5	6.2	>5.2	76	"Zelgerm" steam treatment	6.3	0.7	31
	600 g ETOX m^{-3} 6hr	4.9	1.9	82	5	4.9	2.5	82	"MicroControl" steam treatment	7.4	1.8	30
					9		2.8			6.6	2.6	30
					11		3.4					
	800 g m^{-3} 6hr, 22°C	4.7	2.7	46	4	4.7	2.3	46				
					8		3.3					
Pepper, black	250 g m^{-3} 6hr	7.5	3.4	58	10	7.5	5.7	39	"HF treatment"	7.7	0.3	28
	750 g m^{-3} 6hr		4.0							7.5	0.9	28
	250 g m^{-3} 6hr	7.3	3.3	58	5	7.2	4.1	148	"Zelgerm" steam treatment	7.8	5.8	31
					7.5		4.6					

continues

Table 33-7 Continued

	Ethylene Oxide				Ionizing Radiation				Thermal Methods			
Spice	*Fumig. Condit.*	*Initial Count*[a]	*Lethality*[b]	*References*	*Rad. Dose, kGy*	*Initial Count*	*Lethality*	*References*	*Thermal Method*	*Initial Count*	*Lethality*	*References*
	750 g m^{-3} 6hr		3.9						"EVW" steam treatment	7.1	1.9	30
	800 g m^{-3} 6hr, 22°C	6.8	1.5	46	4 8	6.8	2.3 4.4	46				
Pepper, white	250 g m^{-3} 6hr	5.4	2.5	58	5	5.3	2.4	128	"MicroControl" steam treatment	4.8	>1.8	130
	750 g m^{-3} 6hr		3.4						Continuous "MW system"	7.9	3.3	28
	500 g m^{-3} 6hr	4.4	2.3	58	5	4.7	2.1	43	"HF system"	4.8	0.8	28
	500 g m^{-3} 9hr		2.4							6.0	1.7	28
	500 g m^{-3} 18hr		2.4						"Enclosed MW system"	6.1	2.2	28
Turmeric	1000 g ETOX m^{-3} 6hr	6.4	3.9	98	5 10	6.7	4.7 >5.7	60	"EVW" steam treatment	7.4	4.3–5.1	30

[a] Log$_{10}$ cfu g^{-1}.

[b] Log reduction of CFUs by specified treatments.

Table 33-8 Comparison of Decontamination Efficacy of Various Treatments on the Mold Counts of Some Spices

	Ethylene Oxide				Ionizing Radiation				Thermal Methods			
Spice	*Fumig. Condit.*	*Initial Count*[a]	*Lethality*[b]	*References*	*Rad. Dose, kGy*	*Initial Count*	*Lethality*	*References*	*Thermal Method*	*Initial Count*	*Lethality*	*References*
Cinnamon	1500 g m^{-3} 8hr, 20°–25°C	3.7	>2.7	150	2	3.7	>2.7	150				
					5	5.3	4.3	162				
Fennel	1500 g m^{-3} 8hr, 20°–25°C	3.0	1.2	150	2	3.0	0.8	150				
					4		>1.0					
					6		>2.0					
Fenugreek	1500 g m^{-3} 8hr, 20°–25°C	2.5	>1.5	150	2	2.5	0.7	150				
					4		>1.5					
					5	3.4	2.1	162				
					10		1.8					
Garlic powder	800 g m^{-3} 6hr, 22°C	2.8	0.6	46	4	2.8	1.3	46				
					8		1.8					
					10	3.9	2.9	39				
Ginger	1500 g m^{-3} 8hr, 20°–25°C	3.5	>2.5	150	2	3.5	1.3	150				
					4		>2.3					
					6		>2.5					
Onion powder	800 g m^{-3} 6hr, 22°C	4.2	1.8	46	4	4.2	2.4	46				
					8		3.7					
					2	4.8	1.4	34				
					4		2.1					
					6		2.3					
Paprika	600 g m^{-3} 6hr, 25°C	2.7	0.1	82	5	2.7	>1.7	82	“MicroControl” steam treatment	4.0	>2.0	130
	800 g m^{-3} 6hr, 22°C	2.2	0.5	46	4	2.2	1.0	46	“Zelgerm” steam treatment	3.8	0.3	31
					8		>1.2					
					5	5.7	>4.7	76				
Pepper, black	800 g m^{-3} 6hr, 22°C	4.4	>3.4	46	4	4.4	>3.4	46	“Zelgerm” steam treatment	3.5	1.5	31
					2.5	4.6	>3.6	163	“EVW” steam treatment	4.3	>3.3	30
					5	4.8	>2.8					

[a] Log_{10} cfu g^{-1}.

[b] Log reduction of CFUs by specified treatments.

Table 33-9 Comparison of the Effects of Irradiation and Ethylene Oxide Treatment on Thermophilic Aerobic Bacterial Counts of Various Spices

	Log cfu of Thermophilic Aerobic Bacteria g^{-1}											
		Irradiated at (kGy)										
Spice	*Untreated*	*2*	*2.5*	*4*	*5*	*6*	*8*	*10*	*16*	*Ethylene Oxide Treatment*	*Fumigation Conditions*	*References*
Allspice	6.2	4.8		3.5		2.0	0.6					153
Celery seed	5.1	3.9		2.5		1.8	<1.0					153
Coriander	5.0			2.8			1.5		<1.0			106
Garlic powder	4.4			<1.0			<1.0		<1.0			106
	4.1			3.3			2.0			3.8	800 g m^{-3} 6hr	46
	3.0						<1.0					153
Onion flakes	5.9			3.5			2.2		<1.0			106
Onion powder	2.8			<1.0			<1.0			<1.0	800 g m^{-3} 6hr	46
Paprika	5.5	4.5	2.3	2.3		<1.0						153
	3.2			1.0			<1.0			1.9	800 g m^{-3} 6hr	46
Parsley	2.6			<1.0			<1.0		<1.0			106
Pepper, black	7.5			4.0			1.5		<1.0			106
	7.3		4.5		<1.5			<1.5				124
	6.6		5.7		4.4			3.6				124
	6.2	5.0		3.7		2.5	1.7	<1.0				46
	5.5			2.9			1.2			3.2	800 g m^{-3} 6hr	124
Sage	2.7				<1.5							124

Source: Reprinted with permission from J. Farkas, *Irradiation of Dry Food Ingredients*, p. 28, © 1988, CRC Press.

and salt resistance, and is more fastidious relative to pH, moisture, and growth temperature requirements than that of untreated spices (Table 33-10).[45,47,81]

A *Code of Good Irradiation Practice for the Control of Pathogens and Other Microflora in Spices, Herbs and Other Vegetable Seasonings* has been prepared by the International Consultative Group on Food Irradiation (ICGFI).[66] Although the irradiation of spices and herbs still shows the best results and is the most reliable and versatile technology,[6,57,103] the use of this method remains relatively limited, because of psychological barriers and consequent resistance by consumers (Chapter 4).

33.10.3 Thermal Methods

Because of the heat sensitivity of the delicate flavors and other essential components of many spices and herbs, or some other specific functional properties, a regular heat sterilization cannot be applied. Spice flavor qualities are directly related to volatile oils and/or enzymatically produced flavor components. Therefore, excessive heat, which may denature enzymes or drive off volatiles, must be avoided.[6]

High-frequency and microwave treatments have also little practical utility, because dielectric heating is seriously hampered at the low moisture content of dry commodities.[153] Because of the heterogeneity of the product and the dielectric field, the heating effect is uneven in these materials, which causes adverse changes in sensory quality.[28,29,106]

During the last decade, various other thermal methods have been developed aiming at reducing or eliminating the natural disadvantages of conventional heating. Hot alcohol vapor treatment resulted in the desired antimicrobial effect with whole kernels, ie, whole pepper, but it was not feasible for ground or leafy spices.[106]

The *Sterispice* technique developed in Denmark[100] involves the encapsulation of whole spices with a protein-coating prior to steam heating under pressure. The coating prevents loss of flavors. The heating cycle is different for different spices and takes the heat stability of their flavor components into account.

Table 33-10 Heat and Salt Sensitivity in the Microbial Flora of a Mixed Seasoning Surviving Radiation Treatment

		Log Number of Cells g^{-1} Capable of Colony Formation at Various Salt Concentration Added to the Recovery Medium[a]					
		Unirradiated			*Irradiated at 3 kGy*		
		0.5% NaCl	*5% NaCl*	*10% NaCl*	*0.5% NaCl*	*5% NaCl*	*10% NaCl*
		Water Activity (a_w)					
Heat Treatment at 80°C (minutes)	*Incubation Temperature (°C)*	*0.99*	*0.97*	*0.94*	*0.99*	*0.97*	*0.94*
0	30	6.0	6.3	6.0	3.1	2.0	1.0
	10	5.5	5.5	3.6	2.6	2.2	<1.0
10	30	6.0	6.1	–[b]	2.4	1.3	<1.0
	10	4.6	4.5	<2.0	<1.0	<1.0	<1.0

[a] The mixed seasoning, either untreated or irradiated, was suspended in the ratio of 1:30 in a liquid medium containing various salt concentrations and was subjected to heat treatment at 80°C for 10 minutes or plated without heat treatment. Numbers of colony-forming units were determined by plating dilutions into nutrient agar of identical composition and salt concentration to those of the suspending media. The pH was set at a value of 6.5 and incubation was performed at either 30° or 10°C.

[b] – = not investigated.

Source: Reprinted with permission from J. Farkas, J. Beczner, and K. Incze, Feasibility of Irradiation of Spices with Special Reference to Paprika, in *Radiation Preservation of Food*, pp. 389–402, © 1973, IAEA, Vienna.

Various other, recently established steam heating processes (reported as *MicroMaster* technology in the British literature,[4,5,156] and *MicroControl, EVW,* and *Zellgerm* processes in the German literature[30,31,103,130]) for pasteurization of spices, optimize the processing parameters of batch steam heating for each type of product to ensure low loss of volatiles and minimal color change. The weakest point is still the sensory quality (flavor and color) of leafy products, paprika, and dry vegetable seasonings. Considering the necessity for grinding and blending of steam-treated whole spices, the potential for recontamination compromises somewhat the steam treatment effect, adding the requirement for availability of a *sterile mill* technique.

The *Masterspice* process uses twin-screw extrusion at high temperatures and shear pressures.[151,156] The extruder process is close to an extraction process and its product is completely different from the original spice.[103]

Unlike the situation for the irradiated products, no labeling requirements exist for these alternative methods of decontamination. The Codex Committee on Food Hygiene agreed that consignments of decontaminated spices should be accompanied by a certificate stating the type of treatment to which the product had been subjected.[20] Relevant provisions already exist in regard to irradiated spices in the *General Standard for Irradiated Foods*[18] and for the labeling of consumer products in the *Codex General Standard for the Labelling of Prepackaged Food.*[19]

Labeling of minor ingredients and second generation products produced with irradiated ingredients is a complicated and controversial issue, and views of governments vary significantly from no labeling of "second generation irradiated food," eg, in the United States (US), to labeling of all irradiated ingredients in the food in the case of Denmark. A survey of national regulations, including labeling, has been prepared under the aegis of ICGFI.[66,67]

33.11 MICROBIOLOGICAL CRITERIA FOR, AND MONITORING OF, MICROBIOLOGICAL QUALITY OF SPICES AND HERBS

The safety and spoilage hazards associated with spices added to foods depend on the probability of contamination with disease-causing or spoilage organisms and toxins. Spices are used in a wide variety of foods and are thus capable of introducing microorganisms that may be of no consequence in some products but highly important in others. Therefore, the spoilage and health hazards presented by a spice must be carefully evaluated in the context of the particular use of the spice, with more stringent requirements being reserved for those spices that are added to foods that receive little or no heat treatment, and to cooked foods that are not reheated before eating.[69,110] It is recommended[6] that control of spoilage organisms can be handled strictly between the spice processor and end user for a specific food application and that the count of mesophilic aerobic bacteria should not be used exclusively as a reliable indicator of spoilage potential. According to the Codex *Proposed Draft Code of Hygienic Practice for Spices and Dried Aromatic Plants*,[20] treated and untreated ready-to-eat spices shall be free from *Salmonella* when 10 samples of 25 g are analyzed by appropriate methods of examination ($n = 10$, $c = 0$).

Commodity standards of various countries or organizations usually do not contain specific microbiological criteria for spices and herbs, only macroscopic "cleanliness" specifications. According to the Microbiology Subcommittee of the American Spice Trade Association (ASTA), "routine examination for mold is advised with occasional mycotoxin audits indicated when mold counts exceed typical levels."[6] However, because of the sensitivity of molds to heat and other processing techniques, methods detecting *both* dead and viable biomass of molds, such as the enzyme-linked immunosorbent assay developed by Notermans and coworkers[108,109] give a better index of the "mold history" of spices and herbs than the viable mold counts.

In the spice trade, mesophilic aerobic viable cell counts below a level of 10^3 to 10^4 cfu g^{-1} were traditionally required and accepted as a maximum count of *decontaminated* spices (in German: "entkeimte Gewürze").

33.12 CONCLUDING REMARKS

Most of the world's spices and herbs are grown in tropical developing countries. Under the prevailing conditions of cultivation and harvest, some soilborne microorganisms are inevitably present and inadequate sanitation in primitive harvest, and postharvest conditions may occasionally permit introduction of small numbers of pathogenic bacteria. With demand for herbs and spices increasing, and with today's requirements for safe and consistent food products of high quality, improvement of microbial quality is needed, and the food industry is looking much more closely at the hygiene and safety implications of using these products. In order to conform with the requirements of the food industries in more developed countries, the use of alternative ingredients such as essential oils, oleoresins, and dry dispersed or encapsulated spice extracts will likely grow. Better methods of growing, harvesting, sorting, cleaning, and storage of the raw materials are obviously needed and hazard and control point assessments should support sensible, effective control measures for the spice industry. However, as illustrated by Table 33-2, comparison of microbiological surveys with large numbers of samples in various parts of the world and several years apart,[65,69,113] shows that the ranges and percentage distribution of microbial contamination among samples of specific commodities are remarkably similar and no improvements can be noted. Good manufacturing practice and concepts such as the hazard analysis critical control points must consider the whole process including production of raw materials in their countries of origin. However, the importers' lack of direct control in exporting countries diminishes critical control point opportunities. Therefore, a need for treatments of specific nonextracted spices and herbs to reduce microbiological contamination to a level low enough to satisfy the food industry will remain for a foreseeable time.

The increasing regulatory pressure against the continued use of microbicidal gases and the banning of ethylene oxide treatment in certain regions of the world have given rise to new challenges for alternative treatments. In spite of technical superiority and government approvals, irradiation treatment still has not been widely applied because of fear of adverse consumer reaction. We may well see, however, changes in the consumer's attitude toward food irradiation technologies from which treatment of dry ingredients is one which has the best application potential. A range of new thermal methods are now being promoted. Their general utility is still restricted; however, further optimization of new heat treatment methods and cleaner sources of raw materials brought about by improved techniques at source will result in less severe heat treatment requirements and, in turn, will reduce flavor loss and discoloration.

Antimicrobial activity of some essential oil components of spices has been well documented. Available data suggest that they can be useful adjuncts in food preservation and could have the potential to be used in some food systems to inhibit growth of foodborne pathogens. The intense sensory attributes of essentials oils may be an impediment to their use in some foods. However, this could be overcome if means were found to enhance their activity, for instance, by combination with other preservative factors. A better understanding of the modes of action of the oils may assist achieving these goals. In spite of their "naturalness," the safety concerns of using them need to be investigated further.

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34 Nuts and Nut Products

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34.1 INTRODUCTION

The term *nuts* is commonly applied to a wide range of fruits and seeds, some of which are not nuts in a strict botanical sense.[77] Menninger[65] gives a popular definition as "any hard-shelled fruit or seed of which the kernel is eaten by mankind." Nuts may be used for edible and nonedible manufacturing purposes; for example, in the former category, for production of oils and fats, and in the latter, chemicals extracted are used for plastics, detergents, and so on. The botanical definition, "a hard, indehiscent one-seeded pericarp generally resulting from a compound ovary, as the chestnut or acorn,"[77] is based on morphological features. The acorn, chestnut, and filbert (hazelnut and cobnut) fit both the botanical and the popular definitions of a nut. However, botanically, Brazil nuts are seeds, groundnuts (peanuts) are legumes, and almonds, coconuts, pecans, pistachios, macadamia, and walnuts are all drupes (fleshy fruit with a hard-shelled stone containing the seed).

Nuts originating in temperate zones include almonds, filberts (hazelnuts), walnuts, and sweet chestnuts. Nuts originating in the subtropics include pecans and pistachios, and those originating in the tropics include Brazils, cashews, coconuts, macadamia, and groundnuts. Many are tolerant of a wide range of conditions.

This chapter will use the term "nut" in its broadest sense, but will concentrate on the 11 most important types of nuts or nut products in world production or trade (Table 34-1). The most important nut worldwide is the groundnut. Other commercially important nuts include cashews, filberts, almonds, and walnuts.

Table 34-1 gives production statistics and importing countries for some of the most important traded

Table 34-1 Production and Trade Information for the Eleven Most Important Nuts

Nut	*Botanical Name*	*Production 1994/5 (1000 metric tonnes)*		*Major Producer Countries*	*Main Importing Countries*
		World	*United States*		
Almonds	*Prunus dulcis* (Mill) D.A. Webb	446.3[a,b]	252.6[a,b]	United States, Spain, Italy Portugal, Morocco, Tunisia	Germany, Japan, France, Netherlands, United Kingdom
Brazils	*Bertholletia excelsa* Humb. & Bonpl.	18.0[k]	0[a]	Brazil, Bolivia, Peru, Chile	United States, United Kingdom Germany
Cashews	*Anacardium occidentale L.*	740.0[a,c]	0[a]	India, Brazil, Vietnam, Mozambique	
Chestnut	*Castanea sativa* Mill, *C. mollissima* Bl., *C. crenata* Sieb. & Zucc.	470.0	1–2 (est)	France, Italy, Portugal, Spain, Japan, China	
Coconut (as desiccated coconut)	*Cocos nucifera* L.	228.0[e]	0[e]	Philippines, Sri Lanka, Malaysia, Indonesia (By exporting country)[n]	United States, United Kingdom, Australia, Germany
Filbert	*Corylus avellana* L.	587[a,c]	23.0[a,c]	Turkey, Italy, United States, Spain	
Macadamia	*Macadamia integrifolia* Maiden & Betche	44.0[a,g]	20.4[a,g]	United States (Hawaii) Australia, South Africa	
Groundnut	*Arachis hypogaea* L.	18,000[a,c,f] (est)	1,931.0	India,[d] China, United States, Vietnam, Argentina	Western Europe, Japan, Canada
Pecan	*Carya illinoinensis* (Wangenh.) C. Koch	165.6[a,i]	165.6[a,h]	United States	Mexico, Canada, W. Europe
Pistachio	*Pistacia vera* L.	230.8[a,j]	52.3[a,j]	Iran, United States, Turkey, Syria	
Walnut	*Juglans regia* L.	325.0[a,l,m]	235.0[a,l]	United States, France, India, Italy, Greece	

[a] See Reference[9]
[b] Kernel basis, 7-year average
[c] In-shell basis
[d] Most of India's production is for oil, not edible nuts
[e] See Reference[10]
[f] Edible nut production ~ 10 million metric tonnes, nuts produced for oil ~ 8 million metric tonnes
[g] See Reference[8]
[h] See Reference[11]
[i] See Reference[12]
[j] See Reference[5]
[k] See Reference[13]
[l] See Reference[7]
[m] See Reference[11]
[n] India is a large producer of nuts

nuts. The United States (US) is the principal consumer for most nuts, apart from groundnuts. The countries of the European Union (EU) are also important consumers of most tree nuts, and the United Kingdom (UK) is the world's largest importer of groundnuts. Consumption of most nuts is very seasonal, particularly for tree nuts such as walnuts, chestnuts, filberts, and Brazils, with highest consumption of these nuts around Christmas in Western countries. Many other nuts are consumed all year round, particularly if processed into other products. In the US, Europe, and Australasia, most nuts are used in the food processing industry, as ingredients for bakery products, cereals, ice cream, and sweets. For home consumption, they are processed into salted, dry- or oil-roasted forms, or used raw.

Depending on the type, nuts are either traded in-shell or shelled. Cashews for consumption are always retailed shelled, but can be traded on an in-shell basis, prior to final processing. Walnuts and pistachios are almost always traded and sold for consumption in-shell. Almonds are usually traded shelled, filberts and Brazils shelled or in-shell. Macadamias are traded in-shell, but sold for consumption shelled. Groundnuts are usually traded as "hand-picked selected" (HPS). The economic value of nuts is shown in Table 34-2.

Most nuts are extremely high fat commodities, rich in calories because the edible portion of most nuts has a high protein and polyunsaturated fat content. The exception is the chestnut, which, even if dried, contains only 4.1% fat. The fat content in pecans can be as high as 75%. Almonds, pistachios, and groundnuts also have high protein contents, while chestnuts have very high carbohydrate levels. Most nuts are also excellent sources of calcium, phosphorous, iron, potassium, and B vitamins. The proximate analysis of 11 selected nuts is given in Table 34-3.

34.2 PRODUCTION

Nuts are usually produced in large orchards or plantations, with the exception of Brazil nuts. The US is a major producer of almonds, macadamias, groundnuts, pecans, pistachios, and walnuts. Middle Eastern countries are an important source of warm temperate nuts such as almonds, chestnuts, filberts, pistachios, and walnuts. Commercial harvesting of tree nuts is either by hand, or by using mechanical shakers to remove the nuts from the tree, after which they are caught below. Data have been taken from many texts[36,65,77,111-113] and from sources noted in Table 34-1.

Table 34-2 Value in US Dollars of Some of the Most Important Nuts (estimates published in 1997)

Nut	*Dollars, US, per kg*
Almonds	3.4–6.7[a]
Brazils	3.8[a]
Cashews	3.8–6.1[a]
Coconut, desiccated	0.9–1.2[a]
Filbert	3.08
Macadamia	12.1
Groundnut	0.8–0.98[a]
Pecan	3.0–4.6[a]
Pistachio	4.4[a]
Walnut	2.1–3.6[a]

[a] See Reference[14]

Almonds. Over half of the world production of almonds is concentrated in California, US, in the Sacramento and San Joaquin valleys. Other countries producing almonds include Spain with about 25% of the world crop, Italy with around 8%, and Portugal, Iran, Morocco, and Turkey. US production of almonds is highly mechanized.

Brazil nuts. Brazil nuts have generally proved impossible to bring into domestic cultivation. They are still mainly collected directly from wild trees in the forests of the Amazon basin, in Brazil, Bolivia, Peru, Venezuela, and Colombia. Production levels of Brazil nuts are in decline because of falling tree yields. Good quality Brazil nuts are exported, while poor quality nuts are used for production of Brazil nut oil. Brazil nuts are exported in-shell or shelled.

Cashews. Cashews can grow on poor soils, therefore, in many countries cashews have been introduced to protect coastal sand dunes, to utilize wastelands or other poor soils, and to help prevent desertification. Cashews are a very labor-intensive crop to harvest; production of cashew nuts is therefore mainly from farmer smallholdings, and secondly from trees planted for soil conservation purposes. The remainder of the crop is taken from larger commercial plantations. Approximately half of the world's cashew nut production and processing is centered around India and Brazil. Cashew shells contain the corrosive cashew nut shell liquid (CNSL), which makes processing hazardous. Large quantities of raw nuts are also shipped to these two countries for shelling from other countries that lack the capability to safely process the nut. Mozambique is the third largest producer, and production takes place in Tanzania, which also has processing capacity. There has been an increase in production in Southeast Asian countries, with Vietnam, Indonesia, Malaysia, and Thailand producing 15% of the world total in 1991.[16] Small-scale processing is also carried out in each country by individual producers for home consumption or local sale.

Chestnuts. Several types of chestnut are commercially harvested. These include American, Japanese, Chinese, and European (variously known as the English sweet chestnut, the Spanish chestnut, or the French chestnut) chestnuts. The main production areas of the European chestnut are France, Italy, Portugal, Spain, and the UK. In the US, the Chinese chestnut has been cultivated to replace the loss of the American chestnut, which was affected by a fungus, *Endothia parasitica.*

Table 34-3 Proximate Analysis of 11 Most Important Nuts (per 100 g)

Nut	*H_2O (g)*	*Calories*	*Protein (g)*	*Fat (g)*	*CHO Total (g)*	*Fiber (g)*	*Ash (g)*	*Ca (mg)*	*P (mg)*	*Fe (mg)*	*Na (mg)*	*K (mg)*	*β-Carotene (μg)*	*Thiamine (mg)*	*Riboflavin (mg)*	*Niacin (mg)*	*Vitamin C (mg)*
Almond	4.7	598	18.6	54.2	19.5	2.6	3.0	234	504	4.7	4	773	0	0.24	0.92	3.5	Trace
Brazil	4.6	654	14.3	66.9	10.9	3.1	3.3	186	693	3.4	1	715	Trace	0.96	0.12	1.6	–
Cashew	5.2	561	17.2	45.7	29.3	1.4	2.6	38	373	3.8	15	464	100	0.43	0.25	1.8	–
Chestnut (fresh)	52.5	194	2.9	1.5	42.1	1.1	1.0	27	88	1.7	6	454	–	0.22	0.22	0.6	–
Coconut (desiccated)	3.5	662	7.2	64.9	23.0	3.9	1.4	26	187	3.3	–	588	0	0.06	0.04	0.6	0
Filbert	5.8	634	12.6	62.4	16.7	3.0	2.5	209	337	3.4	2	704	–	0.46	–	0.9	Trace
Macadamia	3.0	691	7.8	71.6	15.9	2.5	1.7	48	161	2.0	–	264	0	0.34	0.11	1.3	0
Peanut (raw, in skin)	5.6	564	26.0	47.5	18.6	2.4	2.3	69	401	2.1	5	674	–	1.14	0.13	17.2	0
Pecan	3.4	687	9.2	71.2	14.6	2.3	1.6	73	289	2.4	Trace	603	130	0.86	0.13	0.9	2
Pistachio	5.3	594	19.3	53.7	19.0	1.9	2.7	131	500	7.3	–	603	130	0.86	0.13	0.9	2
Walnut	3.5	651	14.8	64.0	15.8	2.1	1.9	99	380	3.1	2	450	30	0.33	0.13	0.9	2

Source: Adapted from Composition of Foods, *USDA Agricultural Handbook No. 8,* 1975. U.S. Department of Agriculture.

Coconuts. Coconuts are the fruit of a tropical palm. Wild palms are frequently harvested, however, coconut palms can be successfully grown in plantations. The major producing areas lie between 22° N and S of the Equator, in the humid tropics. More than 80% of world production comes from the Indo-Pacific region, with the Philippines and Indonesia the major countries followed by India, Sri Lanka, and Malaysia. Desiccated coconut production is concentrated in the Philippines, Indonesia, and Sri Lanka.

Filberts (Hazelnuts and Cobnuts). Turkey is the center of filbert production (70% of world production). A further 20% is produced in Italy, and 7% in Spain. Production is coastal-based, often on small farms. In Turkey, intercropping is practiced; sometimes sheep and goats pasture under the trees.

Groundnuts. Groundnuts are synonymous with peanuts, the former term more commonly used in developing countries, the latter in developed countries. All groundnuts are now commercially cultivated. India and China account for over 50% of the world's production, followed by the US. However, very little is exported from China or India because of high levels of domestic consumption. In India, most groundnuts are utilized either for groundnut oil production, or as a protein source in cooking. The US is the world's largest exporter of groundnuts while Western Europe, Japan, and Canada are the major importers. In the US, half of the production is for edible nuts, while 25% are exported and the remainder are crushed for oil.

Macadamia. Macadamia nuts are native to Queensland, Australia, but the major producers now are Hawaii, followed by Australia and South Africa. Other producers include Kenya, Guatemala, Brazil, and Malawi. All macadamias are harvested from commercial plantations.

Pecans. Pecans are native to North America, with the southeastern and southwestern areas of the US being the most important production areas. Other major producers include Mexico, Australia, and Brazil. Pecans are produced mainly in large orchards, though some are still collected by forest dwellers directly from the forest floor in Chile and other South American countries.

Pistachios. Pistachio nuts are native to Western Asia. The major production areas are located in Iran, Turkey, and the US. In the Middle East, pistachios are harvested either from wild trees or from orchards.

Walnuts. Walnuts are indigenous to Eastern Asia, South Eastern Europe, and North and South America—the major producers being the US, Turkey, and China. The Persian walnut is the most important commercial type grown. Black walnuts are produced in the US on large farms. In France, Italy, and the US, production is fully commercial and a high proportion is exported. In Greece and Turkey, much of the production from smallholder farms is consumed mainly by the farmers themselves. Germany and Spain are the main importers of in-shell walnuts.

34.3 PROCESSING/PRESERVATION

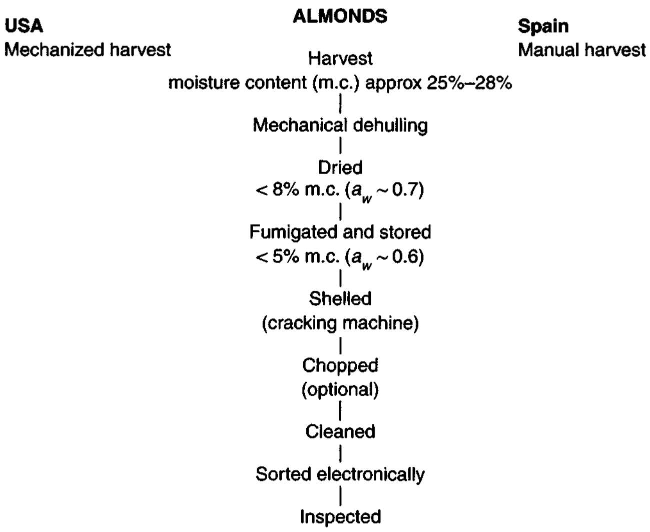
The processing operations for each nut, and for peanut butter are illustrated in Figures 34-1 through 34-12. Moisture contents, where given, are on a "wet-basis" and, with water activity (a_w), are approximate.[15,49] Flow diagrams are generalized. Details can be obtained from many texts,[36,65,77,111–113] unless otherwise stated. General details of processing methods are given below.

34.3.1 Drying

Drying can be carried out under cover, or in the open, to reduce the moisture content of the nuts. In the former case, hot air or radiant heat from a kiln, or solar drying, concentrating the sun's energy and protecting the crop from rain is used. In the latter case, sun drying is used, with no protection from rain. This can be carried out pre- or post-decortication, on the farm or at the processing plant. Groundnuts are windrowed in the field, after lifting plants, which are inverted to prevent pods from touching the soil and to assist them in drying. Pecans, almonds, and walnuts may be windrowed under the trees prior to collection and mechanical drying.

34.3.2 Hulling and Decortication

Hulling refers to the removal of the outer fleshy part of some nuts. Decortication can be used to describe a general process in which a nut or oilseed is cracked open and the kernel recovered, and secondly to an individual stage in a processing operation in which an enveloping layer or hard shell is removed or separated prior to a more involved recovery process. Words such as dehusking, husking, cracking, splitting, and shelling are also frequently used to describe decortication operations. Both operations may be carried out by hand or by machine.



Products:
Natural/blanched/whole/sliced/chopped
Dry or oil roasted/flavored
Almond butter/marzipan/paste
Bakery products

Figure 34-1 Almonds.

34.3.3 Blanching

Blanching refers to the removal of the skin (testa) of the nut, either by wet or dry methods. In the wet method, steam or hot water is used to moisten and swell the skin, followed by rubbing to remove the skin. Blanching can be mechanized, using machines with rubber fingers or rollers, or by hand.

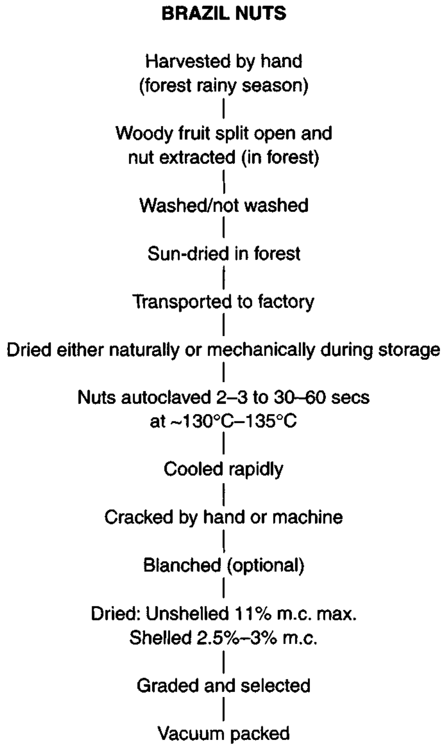
34.3.4 Roasting

Roasting is either carried out dry, using radiant heat, or in oil. Temperatures range from 140°–210°C, for varying lengths of time. Nuts may be dry roasted in air, hot sand, or ashes. Nuts are usually blanched before roasting, to avoid off-flavors from the skin being transferred to the kernel. In some cases, nuts are sand roasted, then shelled and blanched.

Roasting of cashew nuts is a specialized operation because of the presence of CNSL in the shell. Traditional roasting methods allow the oil to escape into the fire and be burnt. Factory-scale methods usually involve collecting CNSL, which is used in industrial processes, including lubricants, brake linings, plastics, lacquers, and antimicrobials.[16]

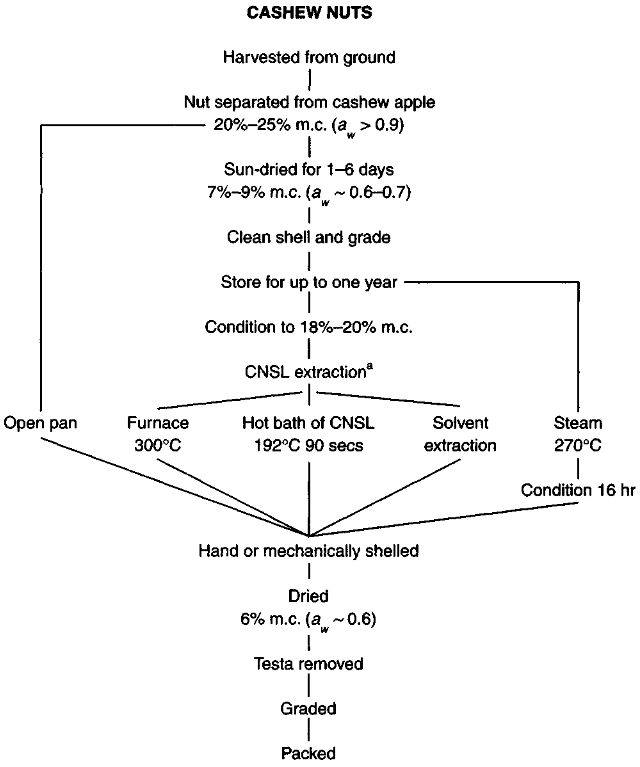
34.3.5 Chopping, Slicing, Shredding, and Grinding

Decorticated nuts, for example, almonds, are sliced and chopped, with or without prior blanching. Desiccated coconut is made by shredding (disintegration) of the pared kernel (meat) of coconuts. The production process includes pasteurization of the coconut meat at temperatures between 70° and 90°C for up to 10 minutes[30,79] either before or after shredding, and may also involve treatment with sulfur dioxide or sodium metabisulphite solution. The product is finally dried to a moisture content of not more than 3% (w/w). Coconut water is the aqueous liquid enclosed by the kernel of the whole coconut, whereas coconut milk is prepared by aqueous extraction of ground-up coconut meat followed by removal of oil by centrifugation. Many nuts are ground, and then mixed with other products to produce flours, pastes, and butters, for example, groundnuts, almonds, pecans, walnuts. Nut oils may also be extracted by grinding, followed by separation processes.



Note: Rancidity is a problem due to high oil content (67%).
Products:
Roasted/salted for bakery/confectionery products

Figure 34-2 Brazil Nuts.



[a]Cashew nuts contain a caustic liquid (cashew nut shell liquid [CNSL]). Care must be taken to avoid contamination of the nut. The liquid is usually removed by the heating process.

Products:
Confectionery/chocolate/salted /roasted
Packed under CO_2

Figure 34-3 Cashew Nuts.

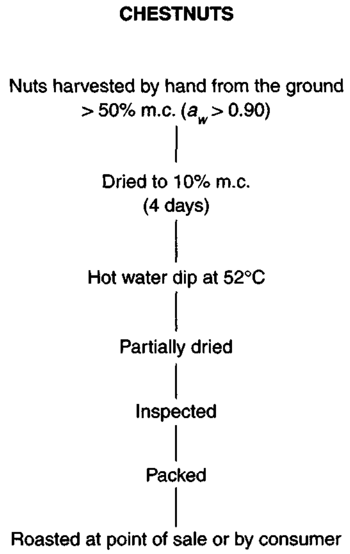
Peanut butter is made from groundnuts by first roasting shelled nuts either by batch or continuous processes. In the former process, nuts are roasted at 160°C for 40 to 60 minutes in a rotating drum, quickly cooled, dry blanched, inspected, then ground and cooled, mixed, and filled into jars. Salt, emulsifiers, and sweeteners may be added at the grinding stage. The product reaches temperatures of 60°–80°C during the grinding process. The final moisture content is less than 2% (a_w <0.1).[49] The continuous process uses a heated airflow over a conveyor belt to achieve the roast.

34.4 INITIAL MICROFLORA

In general, levels of microorganisms on undamaged tree nuts taken at harvest are low because the thick shell of most tree nuts is an important and effective barrier to microbial penetration. The internal surface of a dry, intact nut that has not come into contact with the soil will be virtually sterile. This was demonstrated for coconut by Kajs et al.,[60] by the aseptic removal of flesh and coconut water. Other data have confirmed this for walnuts, pecans, and other nuts.[21,51,64,66] Nuts that become damaged by insects, birds, or poor handling may become contaminated.

It is important to recognize the difference between the more transient surface microflora, and microorganisms that have penetrated the shell and are growing on the kernel. Even if the nut has come into contact with the soil, the internal surfaces may still remain free from contamination. Mold growth on the kernel is unlikely unless the water activity rises to more than 0.7. Appropriate methods should be used to determine the internal and external microflora, by surface disinfection, and the use of semiselective isolation media. In this way, the microbial risks associated with a particular batch can be determined.[78]

A distinction should be made between commercially produced nuts, and those harvested from wild trees. Any nuts allowed to remain in contact with soil for any length of time may suffer insect or vertebrate damage leading to fungal and bacterial penetration. Nuts grown in humid areas are more likely to

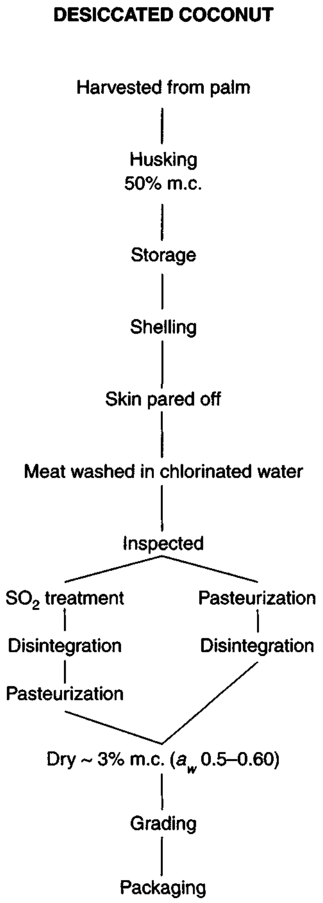

CHESTNUTS
Nuts harvested by hand from the ground
> 50% m.c. (a_w > 0.90)
Dried to 10% m.c.
(4 days)
Hot water dip at 52°C
Partially dried
Inspected
Packed
Roasted at point of sale or by consumer

Products:
Whole dried nuts

Figure 34-4 Chestnuts.

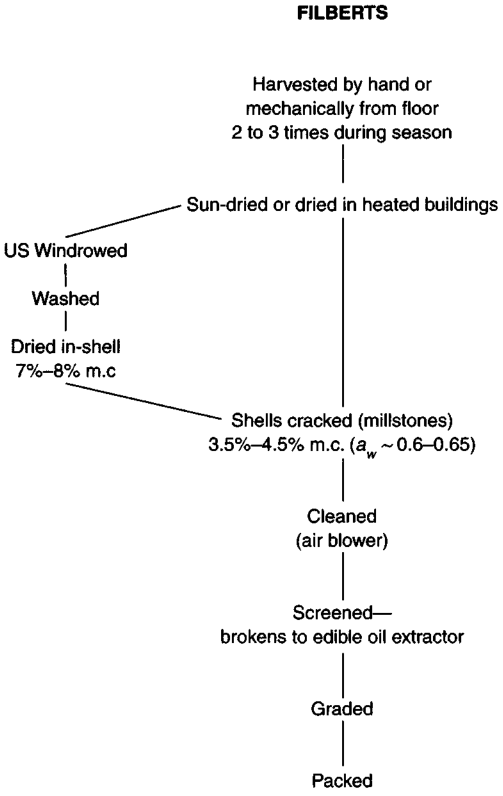
become contaminated with fungi than those harvested from drier areas.

In general, the composition of the contaminating microflora will reflect the soil and air microflora of the region from which the nuts are grown, but in addition, the following common components occur. The aspergilli, in particular *Aspergillus flavus*, are ubiquitous invaders of nuts[73,74] and can produce the mycotoxin aflatoxin. High-fat nut products are particularly susceptible to aflatoxin production. Other important fungi include *Penicillium commune, Penicillium crustosum, Penicillium solitum, Penicillium funiculosum, Penicillium oxalicum, Penicillium italicum, Eurotium* spp., and *Aspergillus versicolor*. Toxins expected include cyclopiazonic acid, penitrem A, citrinin, and sterigmatocystin.[43]



Products:
Various cuts of desiccated coconut from extra fine to sliced or chipped
Sweetened using sucrose, salt, and glycerol
Toasted

Figure 34-5 Desiccated Coconut.


FILBERTS
Harvested by hand or
mechanically from floor
2 to 3 times during season
Sun-dried or dried in heated buildings
US Windrowed
Washed
Dried in-shell
7%–8% m.c
Shells cracked (millstones)
3.5%–4.5% m.c. (aw ~ 0.6–0.65)
Cleaned
(air blower)
Screened—
brokens to edible oil extractor
Graded
Packed

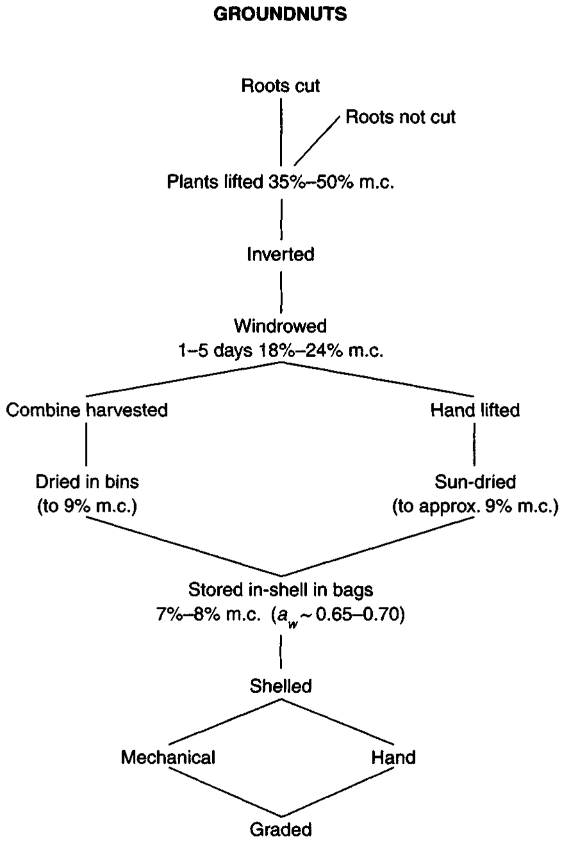
Notes:
Hand harvesting from tree.
Mechanical harvesting from ground.
Products:
In-shell/roasted/salted
Confectionery/bakery/dairy products

Figure 34-6 Filberts.

Bacteria isolated from nuts include *Pseudomonas, Xanthomonas, Enterobacter, Clostridium, Corynebacterium, Bacillus,*[88] *Salmonella,*[48] and *Escherichia coli.*[21] Harvest date can also influence the microbiological quality of the nut. For example, late season almonds can have lower fungal counts than almonds harvested earlier in the season.[62]

34.4.1 Brazil Nuts

Brazil nuts may lie on the forest floor for weeks before they are harvested by forest-dwellers during



Products:
In-shell/dry or oil roasted/salted/confectionery
Flour extraction/sauces-satay

Figure 34-7 Groundnuts.

the rainy season. This can allow extensive fungal and bacterial growth to occur.[77] These nuts are particularly susceptible to invasion by *A. flavus* and *Aspergillus parasiticus*.

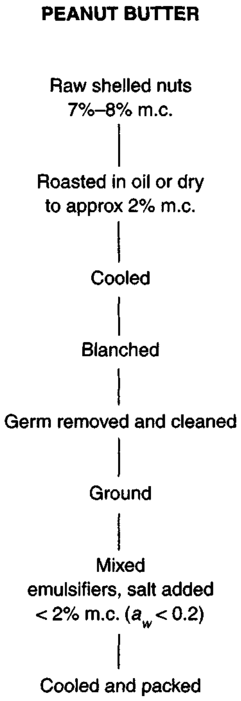
34.4.2 Coconuts

At harvest, nuts are cut from the palm and left to age on the ground beneath the tree where they become contaminated with microorganisms present in the soil. Animal grazing beneath the palms increases the risk of contamination with organisms of fecal origin, eg, *Escherichia coli* or *Salmonella* species. There is a direct relationship between the length of time for which coconuts are left on the ground and the contamination level.[53] Nuts left in puddles of water are more likely to crack, resulting in leakage of coconut water and encouraging growth of a wide range of microorganisms. Spoilage microorganisms that have been associated with coconuts treated in this manner include *Pseudomonas*, *Xanthomonas*, *Bacillus*, Enterobacteriaceae, *Clostridium*, *Corynebacterium*, yeasts, *Penicillium*, and *Aspergillus*.[88]

Microorganisms isolated from the shells of coconuts included large numbers of various microbial types including *Acinetobacter*, Enterobacteriaceae, *Flavobacterium*, *Microbacterium*, *Micrococcus* species, and yeasts and molds.[60]

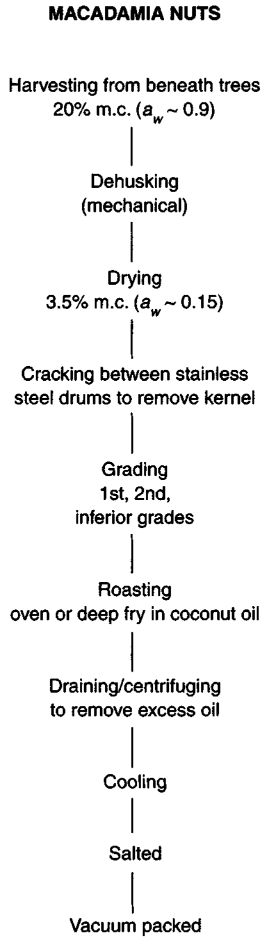
34.4.3 Groundnuts

Groundnuts ripen and mature underground. Therefore, the initial microflora will largely reflect the soil microflora, and contamination is almost inevitable. Sequential harvests of groundnuts in the same soil will increase the level of the soil micro-



Composition:
90% groundnuts minimum
2%–5% salt
Up to 5% emulsifiers and sweeteners
Peanut butter types: fine, medium, coarse

Figure 34-8 Peanut Butter.



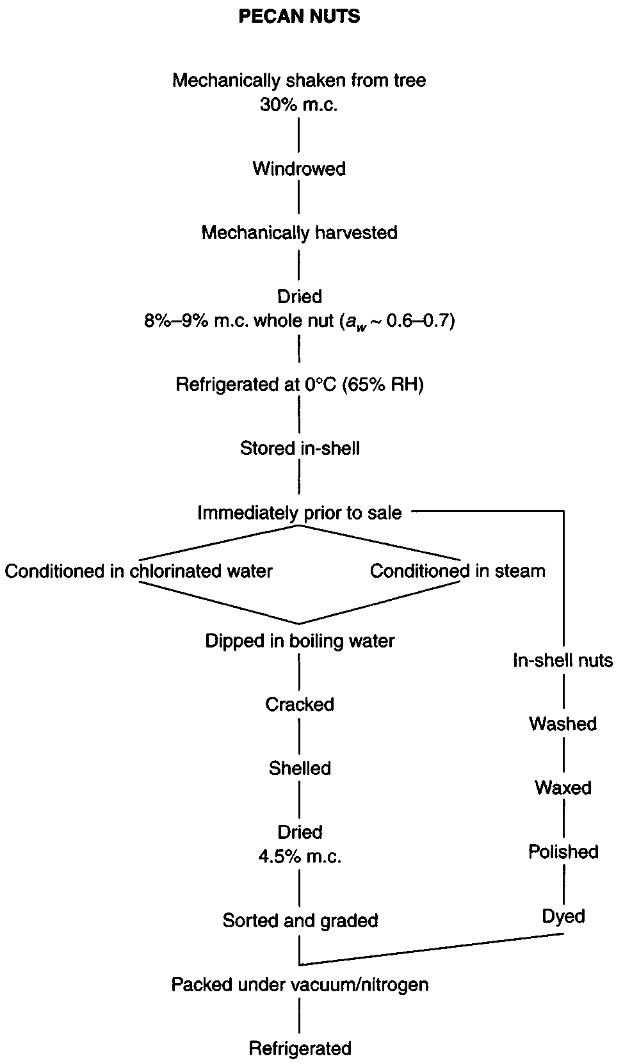
Products:
Dry or oil roasted/salted/baking
Confectionery

Figure 34-9 Macadamia Nuts.

flora.[69,70] Groundnuts are particularly susceptible to drought stress, either caused by low rainfall or overplanting, often associated with sandy soils. This can cause pod cracking, allowing fungi to penetrate the pod and grow within the kernels.

Systemic invasion of groundnuts by *A. flavus* and *A. parasiticus* has been noted under experimental conditions.[72] *Aspergillus flavus*, which is a common component of the soil microflora[58,59] particularly in groundnut fields,[71] has been detected invading plants from contaminated seed and soil sources, growing throughout the plant from emergence onwards into the developing groundnuts. *A. flavus* was more invasive than *A. parasiticus* under these conditions, however, the fungus grows within the plant cells causing little apparent damage.

Some workers have suggested that fungal invasion occurs from the contamination of aerial groundnut pegs or flowers.[50] Factors that can reduce the incidence of fungal invasion and aflatoxin contamination include rotating groundnuts with other crops that decrease the soil populations of *A. flavus*, cultural practices that remove plant residues from around groundnut plants, preventing drought stress, harvesting at pod maturity, and continuous rapid



Products:
Bakery/confectionery/dairy/pecan butter

Figure 34-10 Pecan Nuts.

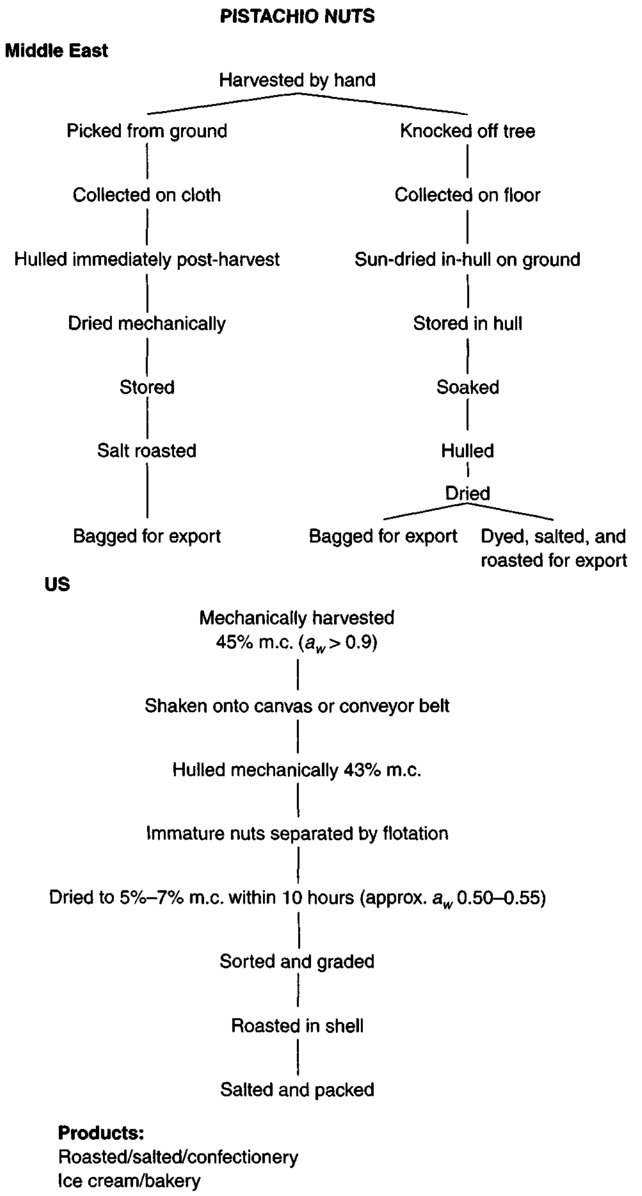

PISTACHIO NUTS
Middle East
Harvested by hand
Picked from ground
Collected on cloth
Hulled immediately post-harvest
Dried mechanically
Stored
Salt roasted
Bagged for export
Knocked off tree
Collected on floor
Sun-dried in-hull on ground
Stored in hull
Soaked
Hulled
Dried
Bagged for export
Dyed, salted, and roasted for export
US
Mechanically harvested 45% m.c. ($a_w > 0.9$)
Shaken onto canvas or conveyor belt
Hulled mechanically 43% m.c.
Immature nuts separated by flotation
Dried to 5%–7% m.c. within 10 hours (approx. a_w 0.50–0.55)
Sorted and graded
Roasted in shell
Salted and packed
Products:
Roasted/salted/confectionery
Ice cream/bakery

Figure 34-11 Pistachio Nuts.

drying of groundnuts after harvest. Resistant lines to fungal invasion have been identified. Resistance factors include tannin content, amino acid components, waxy surface, permeability, and cell structure arrangement.[67]

34.4.4 Pecan Nuts

Pecans gathered as they fall from the tree are virtually mold-free, but if they are left on moist ground for more than 1 week many kernels become infected and visibly moldy.[81] There is a direct relationship between the time of exposure and kernel molding. Kernels from orchards undersown with grass are more likely to become moldy than in those without grass. Typical fungi invading the kernels include *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, *Phomopsis*, *Cladosporium*, and *Alternaria*.[106] Weevil larvae can burrow through pecan shells, providing an entry point and carrying fungal spores into the

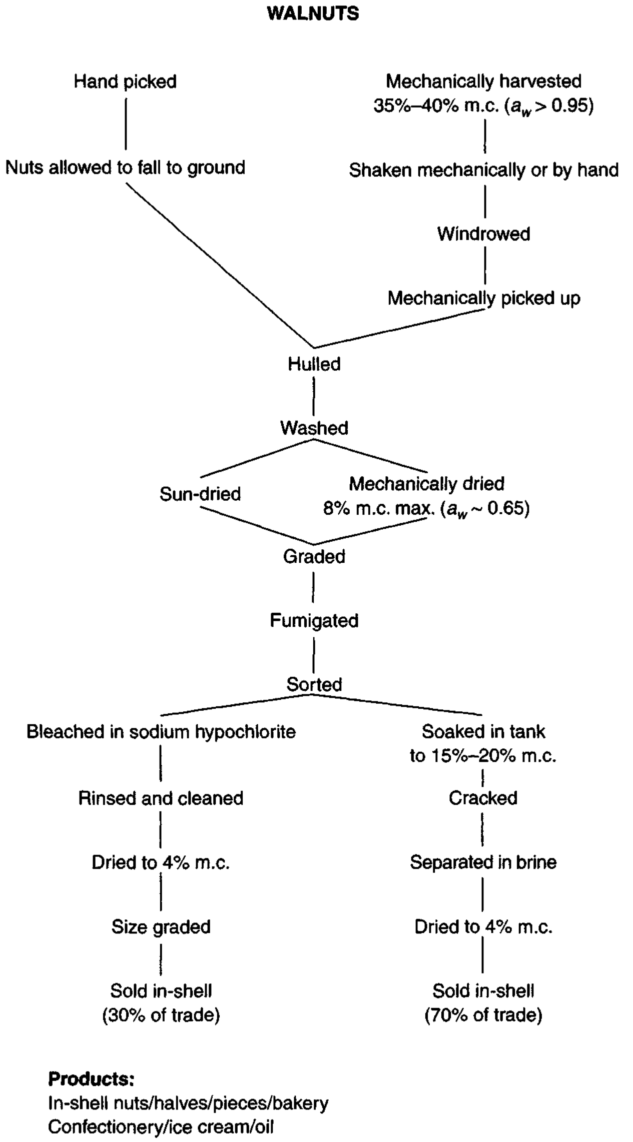

WALNUTS
Hand picked
Nuts allowed to fall to ground
Mechanically harvested 35%–40% m.c. ($a_w > 0.95$)
Shaken mechanically or by hand
Windrowed
Mechanically picked up
Hulled
Washed
Sun-dried
Mechanically dried 8% m.c. max. ($a_w \sim 0.65$)
Graded
Fumigated
Sorted
Bleached in sodium hypochlorite
Rinsed and cleaned
Dried to 4% m.c.
Size graded
Sold in-shell (30% of trade)
Soaked in tank to 15%–20% m.c.
Cracked
Separated in brine
Dried to 4% m.c.
Sold in-shell (70% of trade)
Products:
In-shell nuts/halves/pieces/bakery
Confectionery/ice cream/oil

Figure 34-12 Walnuts.

kernel. Other contamination sources include rainwater and airborne dust.[107]

Nuts collected from grazed orchards were more likely to be contaminated with *E. coli* than samples gathered from nongrazed orchards.[64] Contamination was related to shell cracking. Nutmeats from whole uncracked pecans soaked in a solution containing *E. coli* did not become contaminated. Twenty-four percent of the whole pecans soaked in water for 48 hours to simulate standing in a rain puddle developed openings that did not completely close when the nuts were redried.

34.4.5 Pistachio Nuts

Pistachios have a dehiscent shell within a nondehiscent fleshy hull. If they are allowed to lie on the floor, contamination can result, particularly if hulls also split. Pistachios can also become contaminated on the tree, if the hull splits early, which is atypical (early splits). Fourteen species of *Aspergillus* have been isolated from Californian pistachios, mainly from early splits. *Aspergillus niger* has been isolated from up to 30% of kernels from early splits. *A. flavus* and *A. parasiticus* are three times more commonly isolated from early splits than from smooth hulls.[33] The earlier the hull splits, the more it shrivels and decays.[35] Mold counts in the range of 10^3–10^4 colony-forming units per gram (cfu g^{-1}) have been recorded for pistachios.[52]

34.5 EFFECTS OF PROCESSING AND STORAGE ON NUT MICROFLORA

The microflora of nuts will often change during processing. Some processes, such as blanching and roasting, may lead to a reduction in the microflora, while others such as washing and drying may lead to the ingress and growth of microorganisms on the nuts. Wet blanching will reduce the microbial load more than dry blanching, where mechanical rubbing alone is used to remove the skin. Cutting and grinding operations may spread contamination throughout a batch.

34.5.1 Windrowing and Drying

Groundnuts are dried after harvest by windrowing (stacking in rows) in the field. Windrowing of groundnut plants has been shown to reduce the level of infection of shells and seeds by up to 13% and 36%, respectively. Fungi such as *Chaetomium, Rhizoctonia, Fusarium, Sclerotium,* and *Alternaria* have been isolated from shells of windrowed groundnuts.[76] Pod-contaminating fungi can invade during the drying process. If rain or high relative humidity (>85% RH) occurs in combination with temperatures of between 25° and 35°C, increased invasion of the seed pod can occur. Damaged or cracked pods are more susceptible to fungal attack. The major hazard with groundnuts is the growth of *A. flavus* or *A. parasiticus* and the production of aflatoxins. Drying delays can allow extended growth of the organism and toxin production.

During sun drying, nuts subjected to periods of rainwater may crack, allowing microbial invasion. While the shells of nuts are still wet, microorganisms enter, aided by the water, through small cracks contaminating the internal nutmeat. The nutmeat may still remain wet even after drying the outside of the nut, allowing the proliferation of microorganisms. The nuts may also become contaminated from insects and animals unless pest control measures have been implemented. Fumigation to kill insects will have little impact on any contaminating microorganisms.

Intact coconuts on the tree contain very few microorganisms, however, after harvest the nuts are often stored in unhygienic conditions prior to processing. Microorganisms grow rapidly in the coconut water where damage has allowed this to become contaminated. During drying of desiccated coconut, fungal growth, particularly of *A. flavus,* can be a problem, leading to aflatoxin contamination of the flesh.

34.5.2 Hulling and Decortication

The incidence of *E. coli* on meats of commercially hulled black walnuts depended on the physical condition of the nuts; apparently tightly sealed shells contained only a few *E. coli,* whereas those with visibly separated sutures and spoiled meats yielded the most. Large numbers of *E. coli* on the hulls of walnuts contaminated the hulling water, nuts were subsequently contaminated through separated sutures. Chlorination of the hulling wash water was ineffective. Heating nuts to 100°C in coconut oil, followed by removal of excess surface oil by centrifugation was found to be most effective.[66]

Processing operations during decortication can lead to the transfer of microorganisms from dirty to clean nuts; effective cleaning of decortication equipment is necessary. *Pseudomonas effusa, Corynebacterium paurometabolum, E. coli, Leuconostoc mesenteroides, Proteus vulgaris, Aerobacter aerogenes,* and

Clostridium spp. were isolated from commercially shelled pecan nuts, whereas bacteria were not isolated from aseptically shelled nuts.[21] Similarly, *Penicillium notatum*, *Aspergillus clavatus*, *A. niger*, *Fusidium* spp. and *Trichothecium* spp., were isolated from the commercially shelled nuts, whereas *A. clavatus* and *Trichothecium* spp. only were present on the aseptically peeled nuts. However, fungal hyphae had penetrated both types of nuts.

34.5.3 Blanching and Washing in Hot Water

Blanching processes after decortication will reduce the microbial load considerably. Several nuts including almonds may be processed without any washing or dipping stages, although they will undergo dry blanching. Chlorine in wash water will have little direct effect on microorganisms because of the high levels of organic matter present.

Pecans. Pecans prior to sale are either conditioned in steam or chlorinated water followed by a wash in boiling water. This treatment makes the kernel more pliable and less likely to break during shelling. The US Food and Drug Administration (FDA) requires in-shell pecans to be dipped in almost boiling water for several minutes to destroy *E. coli* and other bacteria. The microbial level will be reduced considerably during these operations; however, some microorganisms may still survive. These treatments may be inadequate to consistently destroy salmonellae in highly contaminated in-shell nuts.[18,21,87]

Other Nuts. Brazil nuts are also boiled in water for short periods and sometimes blanched. Both boiling and blanching will reduce considerably microbial levels. Chestnuts are dipped in water at approximately 50°C, this relatively low temperature is unlikely to affect contaminating organisms to any great extent.

34.5.4 Roasting

The extreme temperatures of dry or oil roasting will reduce the microbial load considerably; however, heat-resistant spores may survive. Some reduction in mycotoxin level may occur; reductions of between 40% to 80% in aflatoxin concentration have been noted for groundnut products[89] and for pecans. Drying also occurs during roasting; further microbial growth post-roasting is unlikely, unless products are stored inadequately.

34.5.5 Chopping, Slicing, Shredding, and Grinding

Cutting and chopping operations will spread contamination throughout a batch; product-cutting machinery and lines must be kept scrupulously clean to avoid this. Contamination with *Salmonella* has been a particular problem in the commercial manufacture of desiccated coconut.[48] According to a report published in 1969,[4] *S. senftenberg* was a frequent contaminant of desiccated coconut. In a survey of foods to study the effect of an improved method for detection of salmonellae, published in 1992, 3 out of 12 samples of desiccated coconut were positive for *S. senftenberg*.[28] A further study of retail samples of many foods in 1990–1991 showed that of 185 samples of imported coconut 4% were contaminated with salmonellae but the serotype(s) present were not reported.[70]

Peanut butter is a shelf-stable product, under normal storage conditions, with a final water activity below 0.2. Roasting and grinding operations heat the nuts, reducing the microbial load considerably, as well as drying the product. Any further microbial growth is unlikely, unless water is allowed to re-enter the retail packs after processing. The heating process may also reduce the levels of any contaminating mycotoxins.[89]

34.5.6 Storage

Groundnuts bulk-stored at ambient temperatures under modified atmospheres accumulated less aflatoxin than those under conventional storage. Other fungi are able to grow and cause physical deterioration of seeds.[108] The survival of *E. coli* on in-shell pecans and its destruction with propylene oxide has been studied.[17] *E. coli* survived longest on low-moisture pecans stored at 0°C. Death was rapid in the nut meat and the inner tissue but not on the shell. The effects of 0–800 μg propylene oxide kg^{-1} at 20°, 30°, and 37°C were evaluated under relative humidities of 0% to 97% for 4 to 16 hours exposure times. Propylene oxide was more effective at increased temperature and relative humidity and longer exposure times. Up to 400 μg propylene oxide kg^{-1} may be required to disinfect highly contaminated pecans. The mold population of almonds may decline during storage at 25°C, but not at 5°C, during storage for up to 385 days. Members of the *A. niger* group survived better than most other organisms, consequently increasing in proportion isolated with time of storage; the greatest decline occurring with species of *Peni-*

cillium and *Cladosporium*.[62] The greatest decline in population occurred during the first 60 days.

34.6 BACTERIAL TOXINS

Two outbreaks of botulism were traced to botulinum type A toxin found in peanuts packed in glass jars. Twenty-three of the 90 bottles of the same lot number in the manufacturer's warehouse also contained the toxin. This was the first case in which botulinum toxin was detected in food in Taiwan.[20] The largest outbreak of botulism in the UK in the 20th century was attributed to hazelnut yogurt. The outbreak occurred in 1989 when 27 people became ill and one died. Toxin production did not occur in the yogurt because of the low pH, but the type B toxin had been produced in the hazelnut puree, which had been inadequately processed before addition to the prepared yogurt.[68]

34.7 MYCOTOXINS

Many reviews and surveys have been published on the incidence of mycotoxins in foods and feeds.[19,29,44,56,75,93,110] Most surveys are not country-attributable, in that they comprise data collected from imported products of undisclosed origin. In addition, a large number of individual studies have been conducted on the incidence of individual mycotoxins in particular crops, and factors that affect their production. A selection of survey data is summarized in Tables 34-4 and 34-5.

34.7.1 Aflatoxins

The aflatoxins, produced by *A. flavus*, *A. parasiticus*, and *Aspergillus nomius* are the most common mycotoxins found in nuts and nut products. Aflatoxins were first detected from samples of groundnut meal imported into the UK.[80] Surveys by the US FDA indicate that aflatoxins have been isolated most commonly and at highest levels from groundnut products and groundnuts, followed by imported Brazil nuts and pistachios.[75] They were found occasionally in US-produced almonds, pecans, walnuts, and sunflower seeds. These surveys did not find aflatoxin in cashews, filberts, or macadamias.

Data collected by the joint Food Contamination Monitoring Programme of the Food and Agriculture Organisation/World Health Organisation/United Nations Environmental Programme (FAO/WHO/UNEP) from a range of countries also indicated that groundnuts and groundnut products were the most frequently contaminated nuts, and to the highest levels,[56] followed by pistachios and Brazils. Other surveys have detected aflatoxins in almonds, Brazils, groundnuts and groundnut products, pecans, pistachios, and walnuts.[92,93,96]

For reasons outlined above, most research and screening studies have focused on aflatoxins in groundnuts. Aflatoxins can be produced in the field, during drying, and under storage conditions. They have been detected in groundnuts from all producing regions.[95] Isolates of *A. flavus* taken from groundnuts have been reported to be more likely to produce aflatoxins than those taken from any other commodity, with up to 96% of groundnuts isolates toxigenic, compared with 49% of sorghum and 35% of rice isolates.[82] A seven-year (1982–1989) survey of aflatoxin in peanut butter taken from retail packs indicated that 74% of 2,510 samples were positive for aflatoxin, with 8.6% of positive samples containing between 20–50 $\mu g\ kg^{-1}$, and 2.2% above 100 $\mu g\ kg^{-1}$.[45] Almost 10% were above the US FDA action level of 20 μg total aflatoxins kg^{-1}, the majority in the years 1986–1987. The authors concluded that their findings could be used, in conjunction with other studies, to support a reduction in the 20 $\mu g\ kg^{-1}$ US action level.

In surveys of pistachios in California conducted over several years in 11 orchards, 60% of orchards had pistachios contaminated with *A. flavus*.[34] Only 0.7% of nuts within an orchard were infected with *A. flavus*, however, and 0.1% with *A. parasiticus*; both organisms mainly associated with early splits. In pistachios, the shells of most nuts split prior to harvest; in early splits, the outer hull also splits open, exposing the kernel to infestation. Aflatoxins were mainly associated with rough-hulled early splits, Navel orangeworm-infested and, to a lesser extent, bird-damaged kernels. Other surveys indicated similar data for Californian pistachios.[91,97] Aflatoxin contamination has also been reported in pistachios from Iran.[41] Aflatoxins can be produced in pistachio hulls, although at lower levels than in kernels.[31,34] This has implications for the use of hulls and other pistachio waste as components of animal feeds. Import data for groundnuts into the US indicate that monitoring and control programs are effective in reducing the risk of contaminated products entering commercial channels, but that contamination can occur in many producer countries. In summary, groundnuts, groundnut products, Brazil nuts, and pistachios represent high-risk commodities for aflatoxin contamination; almonds and pecans a lesser risk.

Table 34-4 Aflatoxin Contamination of Nuts

Commodity	*% of Nuts Contaminated*	*Sample Contamination Level, μg Total Toxins kg^{-1}*	*Country*	*Reference*
Groundnuts	14	7% >20, 273 max.	US imports	110
	10–100	0–95% >26	US imports, 10 countries	56
	7.5	range 98–1056	Jordan	55
		20–35600	Various (S. & C. America, E. & W. Africa, Asia, S.E. Asia)	95 (summary table)
	95.8	2–5091	US	63
	6.3		German imports	61
Groundnut products	32	18% >20, 803 max.	US imports	110
	7.4	range 0.5–55.8	Japan imports	96
	96	8–6280	India	32
		10–26700	Various (S. & C. America, E. & W. Africa, Asia, S.E. Asia)	95 (summary table)
Pistachios	11.1	range 0–200	Pakistan	87
	3.0	range 12–1517, mean 452	Japan imports	96
		5.7% >15	Canadian imports	46
		1146 samples, mean <4, 90% <67	US	56
	6.1		German imports	61
	47.9	max. 35	German imports	109
Walnuts	18.8	range 80–400	Pakistan	87
		1.3% >15	Canadian imports	46
	75	15–25	Egypt	1
Brazil nuts		235 samples, mean <4, 90% <65	US	56
		17926, visibly moldy sample	UK	47
	26.5		German imports	61
	42.2	5–8000	German imports	109
Pecans		max. 840	US	85
	6.3	8–25	US	42
Coconut	20	15–25	Egypt	114
Cashews		8.3% >15	Canadian imports	46
Almonds		1.2% >15	Canadian imports	46
	14.4		German imports	61
		110 one sample	Spain	57
Filberts	90	25–175	Egypt	2

34.7.2 Cyclopiazonic Acid

Cyclopiazonic acid (CPA) has been detected in US groundnut crops.[63] Aflatoxins were detected in both the loose-shell and sound mature kernel (LMK and SMK) fractions, although at lower levels in the SMK portion, whereas CPA was detected mainly from the LMK fraction, at up to 6,525 μg kg^{-1}.

34.7.3 Other Toxins

Ochratoxin A has been detected in visibly moldy[86] and other groundnuts,[105] and in coconut.[114] Penitrem A has been produced in pure culture using isolates of *Penicillium crustosum* taken from pistachio nuts, but not in the natural situation.[37] Sterigmatocystin was detected in pistachio nuts from Californian orchards.[90]

Table 34-5 Contamination of Nuts by Other Mycotoxins

Commodity and toxin	*% of Nuts Contaminated*	*Sample Contamination Level, μg kg⁻¹*	*Country*	*Reference*
Cyclopiazonic acid				
Groundnuts	19	Sound mature kernels, range 32–130	US	63
	77.8	Loose mature kernels, range 32–6525	US	63
Ochratoxin A				
Coconut	12	50–205	Egypt	114
Groundnuts	4.9		German imports	61
Zearalenone				
Walnuts	1 sample	125	Egypt	2
Pecans	1 sample	Detected	US	85
Sterigmatocystin				
Pecans		20,000 (single half kernel basis)	US	84
Alternariols				
Pecans		Alternariol monomethyl ether (AME) and Alternariol (AOH) detected in discolored kernels	US	83

In a study of mycotoxin contamination of filberts and walnuts,[2] although aflatoxin was detected in both walnuts and filberts, and zearalenone in walnuts (Tables 34-4 and 34-5), citrinin, ochratoxin A, patulin, sterigmatocystin, T-2 toxin, and diacetoxyscirpenol were not detected. In another study, almond, cashew nut, chestnut, filberts, pistachio nuts, and walnuts were analyzed for the same range of toxins; aflatoxin was detected in chestnuts but no other toxins were detected in any of the other nut types.[1]

34.7.4 Summary

Aflatoxin production is associated with oilseeds and other commodities with a high fat content; nuts are therefore particularly susceptible. Aflatoxins are mainly produced under warm conditions and, therefore, are more likely to occur in nuts from countries with a warm temperate, subtropical, or tropical climate.

Both the growth pattern of the nut and the agricultural practices of producer regions can influence contamination. For example, nuts that grow in the soil (groundnuts) and those that may remain on the soil after maturity, allowing kernels to undergo wetting and drying cycles (Brazils, some almond crops, some walnut and pecan operations), can become contaminated. Other problems are associated with early splitting hulls or shells, which expose the kernel while on the tree (pistachios and almonds); insect- or bird-damaged nuts, which allow entry points for fungi; and slow drying of nuts once harvested, allowing continued growth of fungi and toxin production.

34.8 MICROBIOLOGICAL QUALITY AND SAFETY

Control of microbial contamination, and the potential production of toxins (particularly aflatoxin) in nuts can be achieved by a selection of strategies. Any form of damage can allow the ingress of spoilage organisms. Preharvest strategies include breeding for resistance to insect pests and to fungal invasion, and, if the fungus penetrates, for resistance to the production of the toxin in tissue; manipulation of the growing environment may be used, including avoidance of drought stress in the case of groundnuts. With tree nuts, delays should be avoided between nuts falling to the ground and being picked up. The drying process should be rapid, and uninterrupted. Mechanical damage should be minimized.

Measures to reject nuts contaminated with aflatoxins usually rely on some form of grading. Nuts that are obviously damaged by insects or vertebrates, or that are moldy or immature should not be harvested. Aflatoxin testing of batches at intake is necessary, and loads should be segregated according to the level of contamination. Stores for nuts preprocessing should be clean and dry. Once shelled, more care is required for storage of nuts as they are more susceptible to fungal and insect attack. It is particularly important to avoid moisture uptake by the kernels. Sorting and segregation procedures are required preprocessing. Color sorting, either electronic or manual, can be used to detect some contamination. Mold-infected nuts are often lighter than healthy nuts; in this case, flotation techniques can be used. The storage environment can be manipulated by the use of modified atmospheres, barrier films, and fumigants.[38–40] Processing operations involving heat (roasting, grinding) may reduce the level of a mycotoxin in a batch.

Control measures to prevent bacterial growth and cross-contamination of processed and raw nuts require procedures for maintaining the sanitary quality of the plant and processing equipment. Of particular importance are procedures involving slicing, chopping, or grating of nut flesh, where contamination of a small proportion of nuts can be spread throughout a batch, if grading procedures are inadequate, and equipment is not cleaned frequently.

Codes of hygienic practice for the production of tree nuts[23] and of desiccated coconut[22] have been published. At harvest, if good manufacturing practices (GMP) are followed, nuts are harvested with minimal damage and dried to a water activity less than 0.70, it should be possible to store them for considerable periods of time, as long as they are not exposed to temperature abuse during storage.[3] If the method of harvesting and subsequent handling were based on the combination of GMP and the hazard analysis critical control point (HACCP)[54] concept, then the quality of nuts and all subsequent products would be improved.

34.9 LEGISLATION

Legislation concerning microbiological or mycotoxin standards varies from country to country. Some countries have horizontal (general) standards, others have vertical (specific) standards for groups of commodities.

34.9.1 Microbiological Standards

UK

The UK does not have specific microbiological standards for nuts or nut products. The Food Safety Act 1990 prohibits the processing or sale of food that is not of the quality demanded by the consumer and prohibits processing food in such a way that it becomes harmful to health. These regulations cover the contamination, poor handling or treatment of food, and food ingredients.

EU

The EU does not have specific regulations relating to the microbiological quality of nuts. General hygiene directives apply to foods in these cases.

US

Specific regulations regarding the microbiological quality of nuts and nut products are not laid down. Compliance Policy Guide 7112.11[102] recommends legal action to be taken when *Escherichia coli* at a level of at least 0.36 per gram (determined by most probable number technique and IMViC confirmed) is found in one or more subsamples of nuts.

Codex Alimentarius

This international body has laid down several standards relating to nuts including general hygiene requirements for unshelled pistachios[24] and grated desiccated coconut.[25] When tested by appropriate methods, these products should be free from hazardous microorganisms at harmful levels, and should not contain substances produced by microorganisms at levels that would be harmful to health. Additional requirements for desiccated coconut contained in the recommended international code of hygienic practice[22] state that the product should be free from *Salmonella* and other pathogenic organisms, and toxic substances originating from microorganisms, with a maximum moisture content not exceeding 3%. A similar code of hygienic practice for tree nuts gives similar recommendations, omitting *Salmonella*.[23]

34.9.2 Mycotoxins

Legislation for mycotoxins, mostly for aflatoxins, exists in 77 countries.[104] At least 27 countries have regulations specifically for nuts or nut products, mostly for groundnuts and groundnut products, and in most cases these are more stringent than regulations for other foods.[93,103,104] If specific legislation does not exist in a particular country, then the legislation for general foods applies. Tolerances generally fall in the range 5–10 μg kg^{-1} for aflatoxin B1, and 10–20 μg kg^{-1} for total aflatoxins, depending on the country. Information on some specific countries or groups is contained below.

UK

The Aflatoxins in Nuts, Nut Products, Dried Figs, and Dried Fig Products Regulations 1992[6] specifically prohibit the import from non-European Community (EC) countries of these products, unless through designated places of entry. The analysis of the import itself must be according to specified sampling and analytical procedures. The maximum permitted total aflatoxin level is 4 μg kg^{-1}, at point of sale. If products are to be processed further then 10 μg kg^{-1} is the maximum import level.

EU

No legislation currently exists for foodstuffs, only for animal feed components.[26,27] However, a working document, Draft VI/1669/97 revision 8, effective January 1999, concerning groundnuts, nuts, and dried fruits, and their processed products has been prepared for discussion. Nuts for direct human consumption may not contain more than 2 μg kg^{-1} of aflatoxin B1, or 4 μg total aflatoxins kg^{-1}. Groundnuts that will be sorted or physically treated prior to human consumption may not contain more than 8 μg kg^{-1} aflatoxin B1, or 15 μg total aflatoxins kg^{-1}. Nuts and dried fruit that will be sorted or physically treated prior to human consumption may not contain more than 5 μg aflatoxin B1 kg^{-1}, total aflatoxins 10 μg kg^{-1}. Otherwise, regulations in individual member countries apply.

US

The FDA has established maximum aflatoxin levels in various nut and nut products in Compliance Policy Guides (CPGs). Accordingly, legal action may be taken if aflatoxin levels above 20 μg kg^{-1} (with aflatoxin B_1 identity confirmed by chemical derivatization) are found in groundnuts and groundnut products, Brazil nuts, and pistachio nuts. This is covered in CPGs 7112.02, 7112.07, and 7112.08,[98,100,101] respectively. Other nuts must comply with the guidelines laid down in CPG 7112.06,[99] which states that legal action is recommended if aflatoxin levels are above 20 μg kg^{-1} (with aflatoxin B_1 identity confirmed by chemical derivatization and negative ion chemical ionization mass spectrometry).

34.10 CONCLUDING REMARKS

The major microbiological problems of nuts occur because of mold and mycotoxin contamination. Bacterial contamination presents fewer problems, and is more common in highly processed nut products, for example, *Salmonella* contamination of desiccated coconut.

Contamination with aflatoxin is the main food safety hazard of nuts. Soil contamination, slow drying, or damage are the major predisposing factors determining if contamination will occur. Nuts that grow in the soil (groundnuts), or may lie on the soil after falling from the trees (pecans, walnuts, pistachios, Brazils), may therefore present the greatest risk. Pistachios are a special case in that the nut kernel can be exposed to fungal attack if the hull splits early or is damaged. Brazils are potentially a high-risk crop, because they are harvested from the floor during the rainy season, taking some time to dry, in conditions of high temperature and relative humidity. If plantation management could be achieved, then this would help to improve the ease of harvesting, and reduce the risk of nuts remaining on the ground for too long.

As analytical methods improve and the detection limits for aflatoxin fall, importing countries tend to set stricter limits for aflatoxin. This has serious implications for producer countries, many of which are developing countries.

Most nuts or nut products are consumed without cooking, which in any case has little effect on aflatoxin. Irradiation procedures are not commonly used, and also have no effect on mycotoxins at practical irradiation doses. Mycotoxins must therefore be removed by other means. Selection and grading procedures are still the most common method of removal of the mycotoxin hazard.

Selection and grading procedures need to be designed for each nut, based on a known defect associated with mycotoxin contamination, so that visual selection or machine grading can occur efficiently. Examples include color sorting for groundnuts. Effective rapid detection methods are required for both fungal and bacterial contamination, so that contaminated nuts can be identified more easily in the factory.

HACCP procedures must be integrated into the whole production, transportation, and processing chain, as has happened for other cropping systems, for example, horticulture. Mold and mycotoxin contamination has usually occurred by the time the product has reached the processing factory; the only effective way to control the hazards and minimize losses for the producer is to identify the Critical Control Points along the whole chain and to implement control measures.

Nuts are relatively expensive commodities and many are rich in micronutrients. The potential for adding value is enormous, particularly in the health food market in the industrialized West. These new products themselves may present food safety challenges that must be met to ensure that foods produced are safe.

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35 Sugars, Honey, Cocoa, Chocolate, and Confectionery Products

Jean-Louis Cordier

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35.1 INTRODUCTION

Sugars, the "sweet" components of plants, are formed during photosynthesis from the three basic elements carbon, hydrogen, and oxygen. Generally speaking, and in particular for consumers, the term sugar is clearly associated with sucrose. Sucrose is in fact widely distributed in nature and therefore easily manufactured in large quantities and frequently used as raw material in food products. Other sugars, however, such as dextrose, lactose, fructose, or polyols, such as mannitol, sorbitol, xylitol, and maltitol, also play an important economic role. Sucrose is manufactured from two main agricultural crops, sugar cane in subtropical and tropical climates and sugar beets in moderate or continental climates. These two sources account for 99% of the worldwide sucrose production, while a small amount is produced from sugar palms (1%), sweet sorghum (0.05%), and maple trees (0.01%).[41]

Mixtures of dextrose (glucose) and fructose (also called levulose) obtained by hydrolysis of sucrose are termed invert sugar. Fructose is obtained from solutions of invert sugar by means of ion-exchange separation or from high-fructose corn syrup after purification. Dextrose is obtained from complete hydrolysis of starch. Polyols are present in fruits, vegetables, and cereals in low concentrations. Extraction is, however, not economical, and the different sugars are manufactured by hydrogenation of easily available carbohydrates, maltitol from high-maltose glucose syrups, sorbitol from dextrose syrups, mannitol from fructose, and xylitol from xylose derived from xylan present in hemicellulose. Lactose or milk sugar is found in milk and produced commercially from whey. Sugar syrups are concentrated aqueous solutions of sugars in liquid form, which are either obtained from cane or beet sugar, from sap of the maple tree, or by acid or enzyme hydrolysis from starches of different plants such as potato, corn, or wheat.

Specifications for sugar, dextrose, lactose, fructose, and for glucose syrup are given in the Codex

Standards 4-1981, 7-1981 or 8-1981, 11-1981, 102-1981, and 9-1981, respectively.[21]

Honey is naturally produced by honey bees, mainly from nectars of flowers. Its sugar composition, consistency, and color vary according to the plant of origin; specifications are given in the Codex Standard 12-1981.[21]

Cocoa powder is prepared by transformation of cocoa press cake obtained from mechanically pressed cocoa nibs or liquor to partially remove the fat. Chocolate is manufactured by mixing one or more of the following base components: cocoa nibs, cocoa mass, cocoa press cake, or cocoa powder with other optional raw materials, such as dairy ingredients, sugar, or flavorings. Standards for semifinished products, such as cocoa nibs, masses, press cake, or powder used to manufacture cocoa and chocolate products, are given in the Codex Standard 141-1983.[21] Chocolate and cocoa represent a rather uniform group of products, which are defined in the Codex Standards 87-1981 and 105-1981.

In contrast, sugar and chocolate confectionery products form a very heterogeneous group of products such as bars, blocks and bonbons, boiled sweets, toffees, fudge, fondants, jellies, pastilles, marshmallows, and chewing gum and also very local and traditional products manufactured in different regions of the world. All of these products are manufactured applying different technologies and a very wide range of raw materials and ingredients.[94] Specifications for composed and filled chocolate and cocoa butter confectionery are provided in the Codex Standards 142-1983 and 147-1985.[21]

A major characteristic of most of the products covered in this chapter is their low water activity (a_w ≤ 0.65), which prevents the growth of pathogens. Spoilage by osmophilic yeasts or xerophilic molds may, however, occur. In view of their properties, which lead to rather different microbiological issues, the above mentioned products will be subdivided into four groups: sugars, sugar syrups, honey, and cocoa, chocolate, and confectionery products. The microbiological aspects will be discussed individually.

Products often categorized as confectionery but characterized by high water activities will not be discussed in this chapter. These include products such as sponge-cakes filled with cream, which are related to pastry and dairy products and have microbiological problems that are quite different from those discussed in the present chapter.

35.2 SUGARS

35.2.1 Composition

Sucrose is a disaccharide composed of one molecule of α-D-glucose linked with a molecule of β-D-fructose. White sugar is purified, crystallized, sucrose (min. 99.7%) with less than 0.1% moisture. Depending on the level of purification, brown or soft sugar contain lower concentrations of sucrose (88% to 92%) and have a higher moisture content (2% to 4.5%). Invert sugar is a hygroscopic sugar composed of equivalent percentages of fructose and dextrose as well as residual sucrose in quantities depending on the extent of hydrolysis.

Sucrose or invert liquid sugars are concentrated aqueous solutions frequently used in the food and pharmaceutical industry. Product composition and characteristics may vary from one supplier to another, but typically sucrose liquid sugars have a solids concentration of 67% to 68% compared with 73% to 77% for invert liquid sugar.

Dextrose or D-glucose is a monosaccharide commercialized as crystallized dry powder (min. 99.5%) either in the anhydrous or the monohydrated form with a residual moisture content of maximum 2% and 10%, respectively. Fructose is a monosaccharide with a keto structure forming very hygroscopic anhydrous crystals and is commercialized as dry powder (max. moisture 0.5%). The polyols sorbitol, mannitol, xylitol, and maltitol are commercialized as crystalline powders. Sorbitol and maltitol are also commercialized as aqueous solutions.

Lactose is a reducing disaccharide composed of one molecule of glucose and one molecule of galactose. Normally the monohydrated α-isomer is produced for commercialization as crystalline powder with not more than 6% moisture.

35.2.2 Initial Microflora

The initial microflora of the different agricultural crops may differ greatly depending on the climatic conditions under which they are grown.

Sugar cane (*Saccharum officinarum)* is grown between 15° and 30° N latitudes, covering parts of Brazil, Cuba, Mexico, South Africa, India, China, Australia, and Hawaii. The ideal climate should be warm (30°C) with an adequate moisture during the growing season and cool but frost-free (10°–20°C) and dry during the harvesting season.[11]

The numbers of microorganisms found on the surface of the cane or in the liquid accumulating in axils of leaves will depend on these climatic conditions and organisms belonging to numerous genera such as *Bacillus*, Enterobacteriaceae, *Pseudomonas*, *Lactobacillus*, yeasts, and molds have been described.[137] More specific studies showed that populations of bacteria and fungi in the liquid found in leaf sheaths were related to its sugar content and pH.[9,38]

Ashbolt and Inkerman[3] have shown that the pink sugar cane mealybug, *Saccharococcus sacchari*, is common in countries growing sugar cane. It is found between the leaf sheath and the stem of the plant and excretes an acidic honeydew (about pH 3) favoring the development of acetobacter-like bacteria (for which the mealybug is the vector) and acidophilic yeasts. After die-off of the insects, less acid-tolerant genera such as *Erwinia* spp. and *Leuconosotoc* spp. become dominant. In the case of heavy infestations by the insect, the high quantities of exudate accumulating in the leaf sheath pocket will lead to problems during sugar production. Damage caused by the cane borer (*Diatraea saccharalis*) results in invasion of internal tissues and heavy multiplication of microorganisms. Of particular importance is *Leuconostoc mesenteroides*, which produces dextran in storage tissues of the plant. Similar observations have been made for cane varieties with growth cracks, with damage caused by overburning before harvest, or in the case of plants suffering from freezing damage.[19]

Sugar beet (*Beta vulgaris*) is the second most important sugar crop and is grown in numerous countries, mostly between 30° and 60° N latitudes. The microflora found on the external part of beets reflects that of the soil and genera such as *Pseudomonas*, *Arthrobacter*, *Erwinia*, and *Streptomyces* as well as mesophilic and thermophilic *Bacillus* spp. and *Clostridium* spp. have been described.[16]

In the case of palms (eg, *Phoenix sylvestris*, the wild date palm) *Lactobacillus* spp. and yeasts represent the major part of the microbial flora in inflorescences. *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Acetobacter aceti*, *Acetobacter rancens*, *Acetobacter suboxydans*, *Leuconostoc dextranicum*, *Micrococcus* spp., *Pediococcus* spp., *Bacillus* spp., and *Sarcina* spp. have been isolated and identified from freshly collected sap.[127] The presence of nutrients and the neutral pH of the sap favor growth and fermentation by these organisms.

In the case of whey used for the production of lactose, the initial microflora depends on the origin of the whey and on the initial processing steps (cheese manufacturing, whey storage, transport, and processing) and is described in the chapter on dairy products.

35.2.3 Effect of Processing

Cane Sugar

Harvesting techniques can vary and mature cane may be harvested as such or after burning to eliminate leaves, manually using cane knives (machetes) or mechanically, sometimes with immediate chopping into pieces or billets of about 30 cm length, thus increasing the percentage of exposed surfaces.[33] The incidence of *L. mesenteroides* increases dramatically after burning or harvesting because of recontamination by harvesting tools or insects such as wasps, attracted by the sap.[9,39,135,136] Development of this and other acid-forming organisms is the major cause of deterioration of the quality of cane sugar resulting from the degradation of hexoses and the production of invert sugar, lactic acid, acetic acid, and dextrans, causing losses of sugar of the order of 0.5% to 1.5% depending on the climatic conditions.[124,138]

Reduction of delays during transport from the field to the factory is the major way of preventing deterioration and thus sugar losses of harvested cane. Trials applying biocides or electromagnetic wave energy to delay microbial growth on and inside the sugar cane stalk were not very successful.[33] Sugar cane is further processed into products such as raw or refined sugar (sucrose) and molasses.[138] A simplified flow diagram summarizing the processing steps from the sugar cane stalks to raw sugar and further to refined sugar is given in Figure 35-1.

Cut cane is shredded and passed through a mill composed of rollers, with extraction water flowing in the opposite direction. The raw juice with a temperature normally between 25° to 30°C, rich in sucrose, inorganic and organic salts, amino acids, micronutrients, and with a pH of 5.0 to 5.6 is an ideal medium for microorganisms. However, normally only a few microorganisms predominate and selective enrichment of *Leuconostoc* spp. and other acid-producing organisms occurs. Development of *L. mesenteroides* leads to the transformation of sucrose into dextrose and fructose and to the production of dextransucrase causing polymerization to dextran. This causes sugar losses, and the polysac-

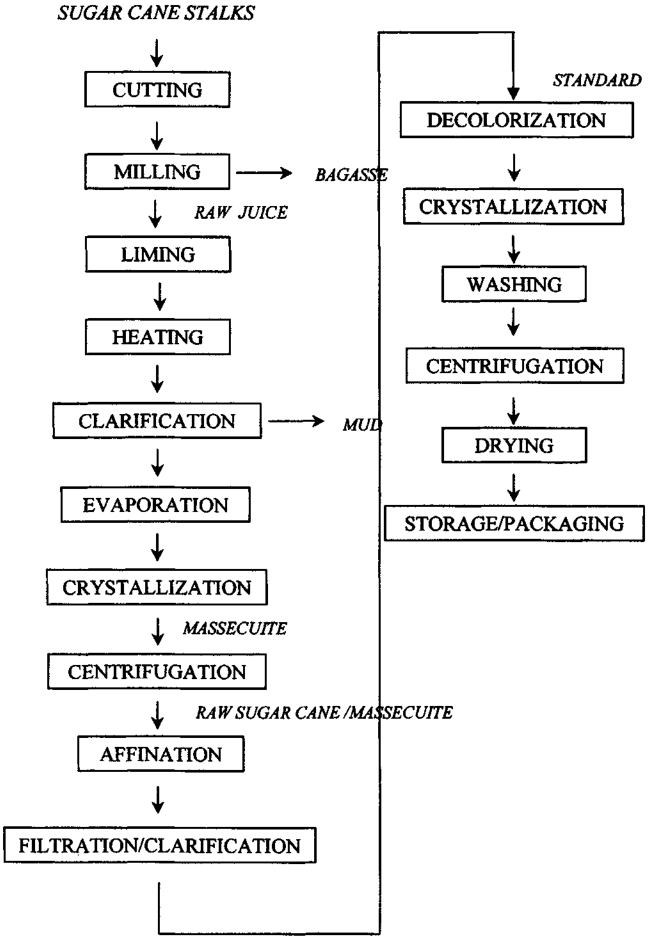

Figure 35-1 Flow Diagram of Cane Sugar Processing.

charide has an adverse effect on further processing steps and on the quality of sugar crystals. Methods for the enzymatic (dextranase) degradation of dextrans already formed by *Leuconostoc* spp. have been successfully developed, allowing the hydrolysis of 72% to 95% of the polysaccharides and improving sugar recovery from contaminated juice.[33]

The extent of recontamination of the raw juice with Enterobacteriaceae or yeasts depends largely on the hygienic status of the mill. Effective housekeeping, sanitation, and use of appropriate biocides are important to minimize sugar losses before further processing.[40] Work has been focused on the inhibition of cane or fungal invertase activity using biocides such as benzoic acid, formaldehyde, or sodium metasilicate.[19,34,57] During subsequent clarification, the pH is raised to 8.0 by the addition of lime and the juice is heated to 80° to 100°C to destroy vegetative microorganisms. Sedimentation and filtration are then used to eliminate precipitates and suspended solids, which are removed in the filter cake mud obtained during pressing and usually recycled. The only viable microorganisms that usually remain in the pressed juice are spores, including those of thermophiles.[19] Residual dextran formed during earlier stages may cause problems during subsequent processing steps, ie, clogging of pipes and interference during evaporation and crystallization of the concentrated juice at 60°C from a supersaturated solution (a_w of approximately 0.85 to 0.88). The concentrated sugar suspension, the so-called massecuite, is cleaned by centrifugation to separate sugar crystals from molasses consisting of sucrose, invert sugar, organic acids, amino acids, nitrogenous compounds, minerals, polysaccharides, and water.

Raw cane sugar used as raw material in the refinery has a pH of 5.0 to 6.0, a water activity of about 0.65, and a sucrose concentration between 95% to 99% with about 0.5% of molasses deposited as a film on the surface of crystals. The microflora of this product consists of spores of *Bacillus* and *Clostridium* spp. that survive thermal processes[19] and osmophilic yeasts and molds. Contamination by these osmotolerant yeasts, such as *Zygosaccharomyces rouxii*, *Hansenula* spp., *Pichia* spp., and *Candida* spp., often occurs in storage tanks for syrup or molasses or by wet sugar residues that support growth of these yeasts in the production lines. Several of these yeasts are also thermotolerant.[2,6,139] Molds such as *Aspergillus* spp., *Penicillium* spp., and *Monilia* spp. are the most common and are often of airborne origin.

Raw cane sugar may occur with a water activity of up to 0.83, which reflects inadequate centrifugation and/or moisture uptake resulting from high humidity conditions. Growth of yeasts is then favored in the molasses film and may cause important economic losses because of hydrolysis of sucrose to form invert sugar. Mold spoilage, however, seems not to be of major concern probably because the oxygen content in the silos used now is normally insufficient to support growth, in contrast with the situation in jute bags, which have been used frequently in the past.

Transformation of the raw cane sugar into crystalline sugar during refining consists of several steps, which may influence the composition of the microbial population of the finished product. During affination raw sugar and molasses are separated by centrifugation and washed with water under high pressure while centrifuging, resulting in the elimination of residual molasses and microorganisms.

The washed sugar is then dissolved in hot water to obtain a syrup of about 70°C/60% solids, mixed with lime and carbon dioxide or phosphoric acid to precipitate impurities, including bacteria, which are then removed by filtration or clarification. Ash is removed by deionization and colors by filtering hot solutions through charcoal beds and ion exchange resins. After evaporation and crystallization, sugar, a product with a low number of microorganisms, <1,000 cfu g^{-1} and frequently <100 cfu g^{-1}, is obtained after drying.[101]

Beet Sugar

Sugar beets are summer or winter crops depending on the climatic conditions of the region. Delayed harvest normally allows an increase in the sugar content because of accumulation of sucrose. However, at the same time the raffinose content begins to increase. Topping (cutting of leaves and crowns), cleaning, and disposal of tops are the major operations involved during harvesting. Harvested beets are then often stored in piles on the fields for between several days and months before further processing. In adequately aerated and covered piles, temperatures of 1.5° to 5°C can be maintained even if the external temperature drops substantially below 0°C. If piles are not well prepared, microbial spoilage may follow damage to the beets, eg, after freezing or overheating (50°C), leading to the production of dextran, levans, or inverted sugars.[22,106] Postharvest losses can be minimized by optimal piling conditions or even by the use of controlled or modified atmosphere storage.[75,76,152] In the case of damaged beets, application of Benomyl® or thiabendazole prevented infection and spoilage by *Penicillium* spp. and *Botrytis* spp.[34] A simplified flow diagram of beet processing up to the raw beet sugar is given in Figure 35-2. Steps applied for the production of refined product are similar to the cane sugar in Figure 35-1.

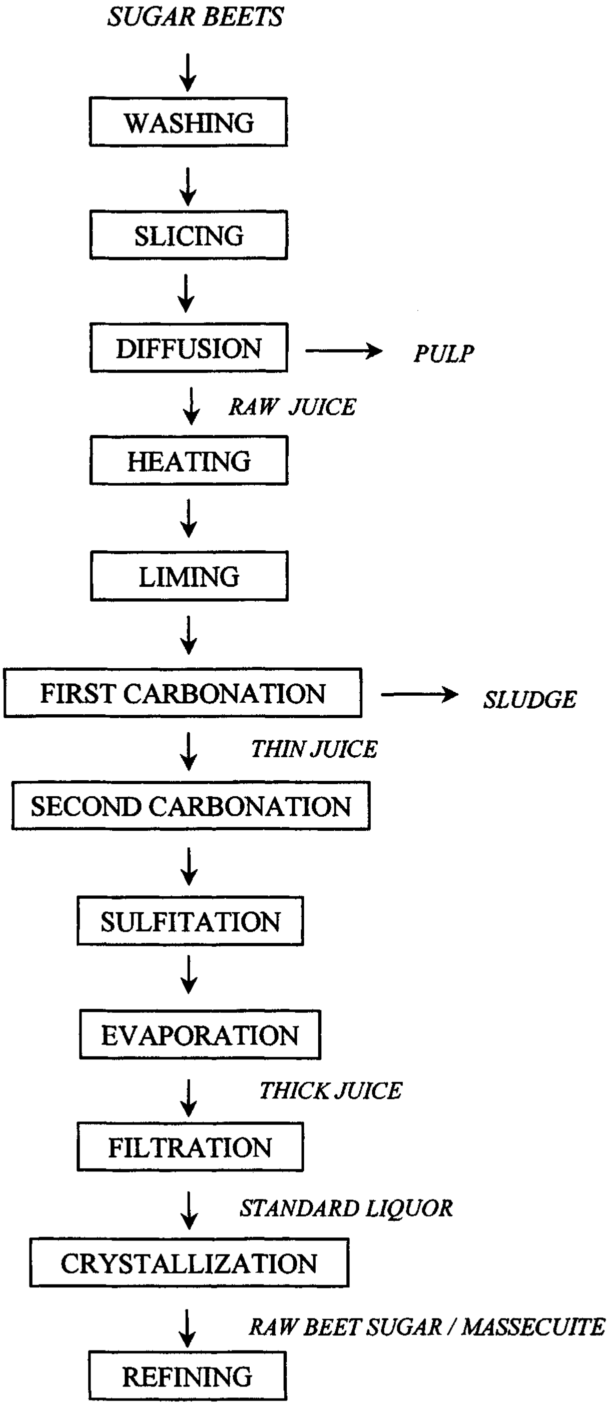

Figure 35-2 Flow Diagram of Beet Sugar Processing.

At the factory, the beets are flumed in water to washers at 30°–40°C. Residual soil is removed in the washers and microbial numbers can be reduced by either using fresh water or by chlorination of recirculated water.[18,96] Leaching of sugar and insufficient removal of some of the soil allow rapid microbial growth. In general the higher the levels of residual soil, the higher the contamination in the juice. Beets are then cut into cossettes or strips and sugar is extracted in diffusers using a counter-current of hot water, either in batch (older) or continuous processes. Diffusers are normally equipped with two ancillary systems, one for the extraction water and one for the juice, to pump, transport, filter, strain, heat, and store the liquids. Details of various beet processing lines are given by Salunkhe and Desai.[124]

Because of their way of operating, various gradients occur in diffusers and their ancillary systems, these include gradients of sucrose (0.5% to 1.5%), invert sugars, minerals, nitrogenous compounds, temperature (25° to 75°C), dissolved oxygen, or pH (5.0–8.0). Microorganisms originating from the beets will therefore be influenced by these ecosystems. The temperature gradient, for example, will favor growth of thermophilic forms between 65° to 70°C, and *Bacillus stearothermophilus* may reach levels of 10^6 to 10^7 ml^{-1} within a few hours[81] resulting in a pH decrease to 5.2 to 5.4.[107] Other thermophiles such as *Bacillus coagulans* occur, while *Clostridium* spp. have been detected in batch diffusers.[7,8,88] The water used for extraction is either fresh water or water recycled from the last extraction step, thus still containing recoverable sucrose (0.5% to 1%). Mesophilic or thermophilic microorganisms may be present, originating from pipes, strainers, filters, tanks, or from diffusion cells. Control can be exerted by heating the water to 80°C or higher, by the use of flocculants or by sanitizing systems with biocides.[14,42] During further processing, the raw juice is heated to 80° to 90°C and lime added in portions as aqueous suspension (milk) or as a slurry of calcium saccharates to precipitate colloidal material. Carbon dioxide is then bubbled through in two stages, the first to facilitate removal of sludge, the second to precipitate residual lime. Precipitates are removed by filtration, and the filtrate (thin juice) is treated with sulfur dioxide to lower the pH and destroy colors. The juice is then passed through ion exchangers, treated again with sulfur dioxide, sometimes filtered through kieselguhr prior to final evaporation (thick juice with a water activity of about 0.88), filtration (standard liquor), and crystallization.[113]

Microbial contamination or recontamination can occur at several steps during refining. Thermophiles are favored in the diluted product during deionization because of the relatively high temperatures. In the charcoal columns, both water activity and the temperature conditions preclude microbial growth, but elution of sugar prior to regeneration of the columns may allow development of thermophilic forms, such as *Clostridium thermosaccharolyticum*, that can produce slimes.[7] Sweet waters originating from bag washers, spillage, wash water from filters, charcoal, and ion-exchange columns may support growth of mesophilic and thermophilic organisms such as *Leuconostoc, Lactobacillus, Streptococcus,* and *Bacillus* spp. depending on nutrients or temperatures.[138,140]

Development of microorganisms, in particular of thermophiles, leads to destruction of sucrose and thus to losses, which may reach up to 1.3%[1] and are more marked in continuous systems.[80] Thus, growth of *B. stearothermophilus* and *B. coagulans*[89] will cause acidification of the juice, from 6.2–6.4 in the beet down to 5.2 to 5.4, mainly because of lactic acid, but also formic, acetic, and glycollic acid.[88,108] Gases such as carbon dioxide, hydrogen, or hydrogen sulfide may also be formed and will influence the extraction procedure.[81] Further effects of microbial growth are corrosion of the equipment, slime formation as a result of synthesis of dextran or levan from sucrose, and transformation of nitrate present in sugar beets to nitrite or nitrogen gas, the first having an impact on the ash content or eventually on the malformation of crystals. The situation concerning the development of fungi is similar to that outlined under Section 35.2.3 for cane sugar.

Liquid sugars are aqueous solutions containing 23% to 34% of water and different types have been developed to meet the needs of customers. They are obtained directly from refiners or, to save transport costs, from intermediate melting stations located near zones with an important number of users. In melting stations sugars are dissolved in water, solutions standardized, and then delivered. These products, however, are sensitive to spoilage and in particular to the development of osmophilic yeasts. Handling under highly hygienic or even aseptic conditions is therefore necessary, and equipment such as pipes, pumps, and tanks have to be designed accordingly. Tanks, for example, have to allow complete drainage and efficient cleaning and sanitation. In order to avoid condensation, ventilation systems that are equipped with absolute filters to remove microorganisms are needed. Normally, tanks are also fitted with UV lamps to sterilize the top, ie, headspace and surface of the tanks.[113]

Other Sugar Crops

In the case of different types of palms, sap is collected from the mechanically tenderized inflorescences or from slits made in the trunk. Microorganisms isolated from sap were identified as *Acetobacter aceti, A. rancens, A. suboxydans, Leuconostoc dextranicum, Micrococcus* spp., *Pediococcus* spp., and *Bacillus* spp., as well as yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.*[127] Lime is added to prevent development of these organisms and fermentation of the sap. After precipitation of the calcium carbonate, the juice is strained and boiled to concentrate it. The syrup obtained is poured into molds for crystallization.[58] The heat treatment causes destruction of vegetative organisms, and the low a_w of the crystallizing syrup (about 0.80–0.83) inhibits growth of other organisms, such as spore-forming bacteria. Moisture uptake may occur in humid conditions and allow the development of molds.

Sweet sorghum (*Sorghum bicolor (L.)* Moench) is either harvested manually using cane knives or mechanically using machines such as corn binders or chopper harvesters. Elimination of leaves, seed heads, and peduncles allows the production of a syrup of higher quality.[23] Processing is similar to that of sugar cane and includes milling, clarification, concentration, cooking, and crystallization.[124] A problem with sorghum juice is the presence of up to 4% starch, which is gelatinized during concentration. Higher concentrations, however, will have adverse effects on the texture of the final syrup.

Dextrose, Fructose, Polyols, and Lactose

As outlined in the introduction, dextrose and fructose as well as the different polyols are derived from syrups, which are prepared as described in Section 35.3. Although not covered in specific publications, microbiological aspects related to the refining process from the syrups to dry crystalline powder are certainly comparable with those of sucrose. Lactose represents a particular case because it originates from milk or whey (45 to 52 g l^{-1}). Industrial lactose is extracted from concentrated whey or from whey permeate; the processing steps related to whey are described in the chapter on dairy products. Extraction of food grade lactose is summarized in Figure 35-3. Although references about this process are not readily available, microbiological problems related to the steps following crystallization are certainly similar to those of other types of sugar.

35.2.4 Pathogens and Spoilage Associations

Although *Clostridium botulinum* has been found in unrefined sugar,[103] this product generally has not been associated with pathogens and has thus never been linked to outbreaks of foodborne disease.

Granulated sugar is normally not a source of spoilage yeasts, unless problems such as moisture uptake cause increases in numbers.[100] Sugar may nevertheless represent a source of bacterial contamination, leading to spoilage of canned foods or bottled drinks. Stringent microbiological standards, in particular for mesophilic and thermophilic spores, have therefore been set by users such as bottlers and canners.[19] The following are examples of specifications.

Sugar for canning purposes:

Samples: 5

Total thermophilic spores: Average of not more than 125 spores in 10 g.

Flat-sour spores: Average of not more than 50 spores in 10 g.

Thermophilic anaerobic spores: May be present in up to three of the five samples, but in any one sample not more than four of six tubes inoculated by standard procedure should contain spores.

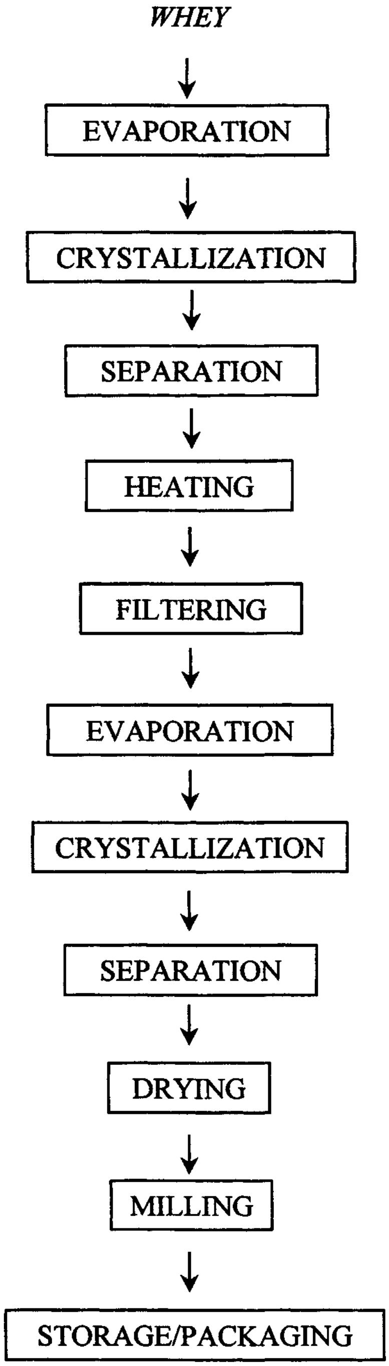

Figure 35-3 Flow Diagram of Lactose Production.

Sulfide spoilage spores: May be present in up to two of the five samples.

Sugar for bottling purposes:
Aerobic mesophilic bacteria: max. 200 cfu in 10 g.
Yeasts: max. 10 in 10 g.
Molds: max. 10 in 10 g.

In the case of liquid sugar for bottling purposes the same standards apply but they are expressed for 10 g of dry sugar equivalent (DSE).

35.2.5 Preservation and Prevention of Contamination

Methods of preventing recontamination, or multiplication during processing have been discussed under Section 35.2.3. The low water activity of granular sugar does not allow microbial growth, and inhibition is guaranteed as long as moisture uptake is prevented. In the case of liquid sugars, preventive measures applied in the frame of Good Manufacturing Practices (GMP) and Good Hygiene Practices are necessary to prevent contamination by and/or growth of osmophilic yeasts. Destruction can be achieved by sanitation and sterilization of the processing equipment and/or of the product, and recontamination during storage can be prevented by fitting tanks with air filters and UV lamps.[40,113] Prevention of condensation by ensuring proper aeration with filtered air is also important to avoid the generation of a_w gradients allowing multiplication of microorganisms in zones with increased a_w values.

35.3 SYRUPS

35.3.1 Composition

Sugar syrups have to fulfill requirements of the users in terms of concentration, composition and ratio of sugars (blends), technological properties, presence of additives.[12,79] They are manufactured for a wide variety of end users and products (eg, bakery, confectionery, drinks, jams, ice cream, meat products, clinical nutrition). These aspects are discussed in detail for glucose syrups by Jackson.[70] According to the Codex Standard 9-1981, glucose syrup should have a minimum of 70% total solids and of 20% dextrose equivalents.[21] Maple syrup is composed of sucrose (88% to 99%) and glucose and fructose (0% to 11%), has a dry matter of 66.5%, and contains several organic acids, minerals, and vitamins.[97]

35.3.2 Initial Microflora

Syrups are manufactured either from cane and beet sugar, from maple sap, or from starches of different origin such as potato, corn, or wheat applying acid or enzyme hydrolysis or a combination of both. The initial microflora of sugar cane and beets is discussed in Section 35.2.2.

Production of maple syrup and sugar is concentrated in the United States (US) and Canada. Sap is drawn from maple trees (*Acer saccharum* or *Acer rubrum*) each year from around mid-February to mid-April. Sap directly withdrawn from the tree is sterile. However, in the sap collected under usual conditions, yeasts and bacteria can be found. In their study, most bacteria were identified by Morselli and Feldheim[97] as *Pseudomonas fluorescens* and their presence was attributed to poorly sanitized collecting devices and tubes. Other studies have shown that the microbial flora of maple sap is related to that found on the trees and isolated from wood and the main organisms belong to the genera *Bacillus* and *Actinomyces*.[114]

Spoilage defects of sap collected under nonaseptic conditions have been described and green sap is due to the development of fluorescent pseudomonads, red sap to red yeasts and some bacteria, milky sap to bacilli, and ropy sap to *Enterobacter agglomerans* excreting exopolysaccharides.[15]

35.3.3 Effect of Processing

Invert syrup is manufactured from sucrose solutions ranging between 60% and 75% total solids, depending on the desired characteristics of the final product. Three techniques can be applied: enzymatic inversion using microbial invertase, acid inversion usually performed with HCl, and ion-exchange inversion on very acidic cationic exchange resins.[113] Most of these processes are carried out at temperatures between 65°C (enzymatic) and 90°C (acid). Starch is used as raw material for the manufacture of other types of syrups such as glucose, fructose, or maltose syrups, which in turn can be transformed into crystallized sugars. The most important types of starch are maize (corn in the US), wheat, potato, and tapioca.

Enzymatic degradation of native starch is very slow. Gelatinization and liquefaction of a starch slurry (30%–40% dry matter) at temperatures of 105° to 107°C are necessary to improve the enzymatic hydrolysis, which is then normally performed at lower temperatures (90° to 100°C) using thermophilic α-amylases of microbial origin.[109] The degree of starch degradation is expressed in dextrose equivalents (DE).

Partial hydrolysates, prepared either by acid or enzyme treatment, can be further treated by one or more enzymes to manufacture a wide range of products. High-maltose syrup is obtained by treating a converted starch slurry with β-amylase or pullulanase and high fructose syrup with an isomerase. High-glucose syrup is manufactured using amyloglucosidase and also serves as an intermediate to prepare high-fructose syrup. Maltodextrins are produced by stopping the conversion process at an early stage, while hydrogenation is used to prepare polyols from different sugar syrups.[82]

After conversion, syrups are filtered, decolorized, concentrated, and, if necessary, purified on ion exchange resins. Complete technical details on characteristics and manufacturing of starch hydrolysates are given by Blanchard and Katz[12] and Olsen.[109] Precise microbiological data on the manufacture of the different types of syrups are not easily accessible and the number of publications is very limited. Because of the conditions of processing no major change in microflora is likely to occur, except the destruction of vegetative organisms and of some spores. Depending on their sugar content and the type of sugar(s), syrups have water activities ranging between 0.70 and 0.85. They are therefore prone to spoilage by osmophilic yeasts such as *Zygosaccharomyces bailii* and *Z. rouxii*, which are able to grow at a_w values as low as 0.65.[142] Spore-forming bacteria may originate from the raw materials, starch being a possible source of thermophilic spore formers in canned foods.[63]

As in the case of liquid sugar, recontamination may occur during intermediate storage in tanks, and during transport in pipes, pumps, or trucks. The installation of absolute air filters and UV lamps as well as adherence to GMP are also recommended to prevent recontamination.[113]

Maple sap is an aqueous solution containing 1% to 9% sucrose,[97] which is concentrated to prepare maple syrup and sugar. Concentration is usually performed in special pans and syrup is then further transformed into hard and soft sugars in molds, the typical flavor and color being developed during heat processing. Normally 40 liters of sap are necessary to produce 1 liter of syrup. Details of preparation of different derivatives are given by Salunkhe and Desai.[124]

35.3.4 Pathogens and Spoilage Associations

The situation regarding pathogens is identical to that for sugars (Section 35.2.4) because this group of products has never been linked to outbreaks of foodborne disease.

The requirements for specifications regarding spore formers are similar to those outlined for sugars, in particular for syrups used in the bottling industry.[19] However, contrary to the situation in granulated sugars, the presence of osmophilic yeasts such as *Z. rouxii*, *Z. bailii*, or *Saccharomyces cerevisiae* is detrimental either to the syrup itself or for products manufactured using these syrups. Development in the product itself may lead to deterioration of organoleptic or textural characteristics, while contamination of confectionery products, for example, will cause fermentation, gas production, leakage, and product loss.[117]

The presence of *Clostridium botulinum* has been reported in 13 of 1,010 samples of corn syrup.[77] In the positive samples, levels of approximately 50 spores g^{-1} were found by MPN. The organism was not found in two further surveys, nor in 43 samples of several types of syrup[62] or 738 samples of corn syrup or products containing corn syrup.[85]

35.3.5 Preservation and Prevention of Contamination

Recontamination with osmophilic yeasts can be minimized or controlled applying the same measures as outlined under Section 35.2.5. In the case of the different types of sugar syrups, preventive measures applied in the framework of Good Manufacturing Practices and Good Hygiene Practices are necessary to prevent contamination by and/or growth of osmophilic yeasts. Prevention of condensation by ensuring proper aeration by filtered air is important to avoid generation of a_w gradients, allowing multiplication of microorganisms in pockets or surfaces with increased a_w values. In the case of maple syrup, destruction of microorganisms can be achieved by sterilization of the processing equipment or of the product, eg, before packaging.[37] Other techniques have been applied such as UV irradiation of surfaces of syrups during storage.[36,52]

35.4. HONEY

35.4.1 Composition

Honey is essentially composed of water (average 17%) and of the two sugars fructose (average 38%) and glucose (average 30%), other sugars including sucrose and maltose amounting to up to about 13%. Furthermore, organic acids, minerals, proteins, amino acids, and enzymes can be found. The composition of a honey sample will depend mainly on the type of nectar(s) and characteristic differences can therefore be observed. A compilation of the average composition of several types of honey is provided by White.[149] The water activity varies between 0.5 and 0.6 and the pH between 3.4 and 6.1 with an average of 3.9. The internationally accepted detailed norms are given in the Codex Standard 12-1981.[21] Different commercial forms are found, the traditional liquid or crystalline products or a mixture of both; solid crystallized or granulated honey resulting from glucose crystallization; the easily spreadable creamed honey obtained by a physical process. Comb honey and chunk honey are frequently seen, where combs are either commercialized as whole sealed pieces, sections of them, or as pieces added to liquid honey.

35.4.2 Initial Microflora

As outlined by Snowdon and Cliver[130] in their thorough review, microbes of interest to the honey processing industry are those able to withstand the specific characteristics of honey such as sugar, acidity, and presence of antimicrobials. The microbial content of honey is generally low and several surveys[130] have shown counts of <100 cfu g^{-1}, exceptionally of up to 1,000 or 10,000. *Bacillus* spp. have been identified as the dominant flora in most studies. The microorganisms found in hives come from the bee, the nectar, and pollen as well as from external sources. *Bacillus* spp. have been predominant isolates from feces of bee larvae[49] followed by Gram-variable pleomorphic bacteria, to a lesser extent by molds, *Actinomycetes*, Gram-negative rods and yeasts, whereas *Clostridium* spp. have not been found. This is in agreement with other studies on adult honey bees.[50,51] Because nectar is almost sterile,[48] it is suggested that pollen may be the main source of the intestinal flora. Secondary sources of microorganisms are humans, equipment, dust, insects, animals, water, and sweeteners. Comparison of microbial isolates from bees and honey[130] shows that certain intestinal organisms of the bee do not survive in honey. Vegetative bacteria, such as *Gluconobacter* spp. and *Lactobacillus* spp., present at initial stages, disappear during ripening of honey because of a drop of the water content to below 18%.[123] Two spore formers, *Bacillus larvae* and *Clostridium botulinum*, can be found at low frequencies in honey. The first is the cause of the "foul brood" disease of bees and has therefore an economic impact on honey production.[65] The isolation of *C. botulinum* from honey has been described by several authors[62,66,131] and has been related to outbreaks of infant botulism.[91] *C. botulinum* spores can survive for rather long periods in honey[104] and their increased incidence at higher levels seems to be linked to the presence of diseased bees in hives, favoring multiplication and sporulation.[102]

Molds are detected regularly in the intestinal content of bees and in the environment of hives. Molds are normally found in honey at low levels of 1 to 100 cfu g^{-1},[116] or 250 cfu g^{-1},[146] and widespread genera such as *Aspergillus* and *Penicillium* are usually found.[49,147] Xerophilic forms able to grow in honey have not been reported, suggesting that molds tend to survive but not to develop.

In the case of yeasts, *Saccharomyces* spp. are predominant[145] but other genera have been reported[130] including *Rhodotorula, Debaromyces, Hansenula, Lipomyces, Oosporidium, Pichia, Torulopsis, Trichosporon, Nematospora, Schizosaccharomyces, Schwanniomyces,* and *Zygosaccharomyces.* The concentration of yeasts is frequently proportional to the moisture content[27] and yeast counts ranging from 0 to 10^5 and even 10^6 g^{-1} have been reported.[55,122] Osmophilic yeasts, such as *Zygosaccharomyces,* found in honey, may originate from flowers and soil, other types from combs, air, and equipment.[122] *Z. rouxii* and *Z. bisporus* have frequently been isolated from honey[71,117] and high levels resulting from multiplication will obviously cause fermentation and lead to unacceptable organoleptic changes.

35.4.3 Effect of Processing

Heating is an essential factor in the processing of honey, but overheating must be avoided to minimize formation of undesired compounds such as hydroxymethylfurfuraldehyde (HMF) or color changes.[134] First, frames are stored at 32° to 35°C for about a day to improve extractability. With few exceptions, honey is extracted from decapped combs after removal of the wax layer covering the cells. Uncapping is performed either manually by means of a hand knife dipped into hot water, by electrically heated knives, or by automatic systems allowing several frames to be uncapped at the same time. Wax residues generated during this operation are separated by skimming, settling, or melting.

Extraction is performed by centrifugation, spinning the honey out of cells and then recovering the honey into sump tanks, which are often jacketed with warm water. Warming is then necessary to overcome cooling (through evaporation) that occurs during extraction, and heating to about 46°C in the sump tanks improves the subsequent settling and straining steps. The extracted honey is then strained using different types of strainers, from simple straining cloths to larger systems equipped with screens of different meshes. Before packing, honey is usually stored in bulk in tanks or drums and liquefied before repacking. Liquefying is normally performed in melting rooms heated by hot air (60° to 70°C).

The moisture content is a major parameter governing the keeping quality of honey. Honey is hygroscopic and may absorb considerable quantities of moisture if not stored under appropriate conditions. An optimum of 17% to 18% moisture can be maintained by controlling the environmental humidity at about 60%. Details on processing are given by Crane.[27]

35.4.4 Pathogens and Spoilage Association

The only pathogen of concern for public health is *C. botulinum,* which has been isolated from commercial samples (Section 35.4.2) (see Chapter 41). The occasional presence of Enterobacteriaceae is due to contamination during extraction and manipulation of honey.[17,116] As outlined under Section 35.4.2, osmophilic yeasts may cause spoilage at slightly increased water activities[126] or spoilage of confectionery products containing contaminated honey. Spoilage of honey by *Z. bailii* may occur at water activities ranging between 0.65 and 0.68.[143] Heat treatments at temperatures >60°C provide a killing effect despite the fact that the low water activity has a protective effect on the yeasts. Bacterial spores, however, will normally survive this treatment.

35.4.5 Preservation and Prevention of Contamination

Heating represents the only means of destroying osmophilic yeasts. Water activity is otherwise a good barrier and multiplication, in case of presence, can be inhibited by ensuring that it is kept at 0.65 or below, by avoiding moisture uptake (hygroscopicity, condensation). Postprocess contamination during handling of honey, originating from equipment or air in contact with the product, can be minimized or eliminated by applying Good Hygiene Practices.

Contamination by *C. botulinum* cannot be prevented and normal processing conditions are not designed to eliminate spores. Destruction can only be ensured by sterilization, preferably of diluted honey. Cases of infant botulism can be avoided by applying the recommendations issued by the American Academy of Pediatrics not to feed infants under 8 to 12 months old products with honey or products containing untreated honey (eg, water).

35.5 COCOA, CHOCOLATE, AND CONFECTIONERY

35.5.1 Composition

During processing of cocoa beans, several intermediate products are obtained that can be used to manufacture cocoa and chocolate products. Cocoa nibs are obtained from clean cocoa beans after mechanical breaking. Grinding of nibs allows production of a liquid cocoa mass, which can be separated under pressure into cocoa press cake and cocoa butter. Descriptions and detailed compositions of these intermediate products as well as others are given in the Codex Standards 86-1981, 105-1981, and 141-1983.[21]

Chocolate is a homogeneous product prepared from one or several ingredients mixed in different ratios, depending on the type of chocolate: cocoa butter (12% to 58%); cocoa solids (0% for unsweetened chocolate; otherwise 20% to 35%); milk fat (≅0.5% to 7%); fat-free milk solids (3 to >14%); and sugar (<55%). The precise definitions and composition of the different product categories are given in the Codex Standard 87-1981.[21]

In the literature and articles for laymen or consumers simpler descriptions may be found. "Milk chocolate" is composed of milk solids, milk fat, cocoa solids, cocoa butter, lecithin, and flavors as opposed to "dark chocolate," where the milk components are omitted and "white chocolate," which does not contain cocoa solids.

Confectionery products are very heterogeneous and will not be discussed here. Descriptions and definitions of certain products can be found in the Codex Standards 142-1983 and 147-1985.[21]

35.5.2 Initial Microflora of Cocoa Beans

The fruits of the cocoa, *Theobroma cacao* L., commonly referred to as pods, are removed from the trees by cutting the fruit stalk with a sharp instrument. Each pod contains about 30 to 50 beans (seeds) embedded in a sterile, acidic mucilaginous pulp (pH 3.6–4.0), rich in water and sugars.[10] The raw beans, composed of two cotyledons, a germ (radicle), and a seed coat (testa) contain about 30% water and 30% proteins, starch, sugars, phenolic, and other organic compounds.

The pulp and beans are contaminated during and after breaking of the pods by numerous external sources such as hands and tools of harvesters, earth, leaves, wood from containers, insects, and animals.[90,112] During fermentation, pulp residues are degraded by external microbial enzymes and internal enzymatic processes, leading to the synthesis of flavor precursors. Heat and acids formed during this phase also cause destruction of the germ, thus avoiding degradation of cocoa fat normally associated with germination.

The beans are allowed to ferment by applying very different techniques, depending on the region, and methods include fermentation in piles, heaps, baskets, wooden boxes, or trays.[128] Despite problems linked with the heterogeneity of the fermenting mass, changes resulting from mixing, and the different sampling methods applied, three different phases have been identified.[60,112] In most cases, succession of different microorganisms is not clear-cut and transition phases or overlaps occur.

Phase 1: During the first 1 to 2 days, yeasts form the predominant population. They metabolize sugars rapidly, in part to ethanol, and excrete pectinolytic enzymes, which break down the mucilaginous gel of the pulp. This facilitates draining of liquid from the pulp, the so-called sweating fluids. Subsequent catabolism of citric acid leads to an increase of the pH from 3.6–3.7 to 4.0.[45,125]

Phase 2: In the collapsed pulp, fermentative yeasts decline by the third day. During this phase, the low oxygen concentration or, if oxygen is present, the high carbon dioxide level, favor the development of lactic acid bacteria. Both homofermentative types, producing lactic acid as the main product, and heterofermentative ones, producing several products, with lactic acid making up 50% or more of the total, occur.[115] Aeration of the fermenting beans favors the development of acetic acid bacteria, which transform ethanol to acetic acid, which is, along with lactic acid, further oxidized to carbon dioxide. The oxidation gives rise to a temperature increase to 45° to 50°C.

Phase 3: These conditions and the steadily increasing pH favor the development of thermophilic spore-forming bacilli. Tetramethylpyrazine, an important component of cocoa flavor, is synthesized by *Bacillus subtilis*.[111,155]

The microflora of fermented beans is representative of the microorganisms of the successive populations and is composed of yeasts, acetic acid and lactic acid bacteria, and *Bacillus* spp., as well as contaminants or spoilage organisms originating from the environment.[112] Spoilage of fermenting beans because of the development of *Aerobacter* spp. and *Pseudomonas* spp. has been observed at pH values above 5.0. Molds may grow on the outer surfaces of beans if regular mixing is not ensured.[121] Penetration of the beans, in particular by *Aspergillus niger, Aspergillus flavus, Penicillium* spp., and *Mucor* spp., however, may occur if sufficient oxygen is available, causing changes in the fatty acid profiles.[59,61] The presence of mycotoxins has, however, only rarely been reported in cocoa.[83] This is probably due to elimination of shells during further processing or to the inhibiting effect of caffeine in beans, where it may be present at concentrations of 0.3 to 3.6 mg g^{-1} depending on their origin.[84,153]

Fermented beans are then dried, either artificially or naturally, to reduce the water content to 6% to 8%. The microflora of dried beans is composed mainly of mesophilic and thermophilic spore formers, followed by vegetative organisms, such as *Enterobacter* spp., *Flavobacterium* spp., and *Micrococcus* spp. as well as yeasts and molds.[5,60]

Subsequent handling, transport, and storage of dried beans may lead to spoilage by xerophilic molds in the case of damaged or improperly dried beans (moisture uptake above 8%).[60] Mold growth is often linked with the occurrence of off-flavors because of lipolytic activity or the excretion of metabolites dissolving in the fat phase.[61,78]

35.5.3 Effect of Processing

A very general flow diagram of chocolate processing is given in Figure 35-4. Numerous alternatives may exist, depending on the manufacturer. The vast majority of microorganisms are found on the outer surface of undamaged beans. Considering their origin and the way in which raw and fermented beans are handled, the presence of *Salmonella* is also likely. Roasting represents the only processing step allowing destruction of the pathogen. The heat treatments are also essential steps in the development of chocolate flavor, initiating important chemical reactions.[154]

Before roasting, raw fermented beans are cleaned to remove extraneous materials. Different roasting processes have been developed including roasting of whole beans, of nibs obtained by breaking beans in winnowing machines, or of cocoa liquor obtained by passing cocoa nibs through grinders and refiners.[87] Temperatures applied during roasting depend very much on the type of cocoa processed and the organoleptical profile desired by the manufacturer. Initial moderate temperatures eliminate excessive water and distribute moisture evenly in the beans, temperatures ranging between 100° and more than 200°C can be applied at later stages. Temperatures of about 105° to 150°C are sufficiently high to destroy vegetative microorganisms, including pathogens

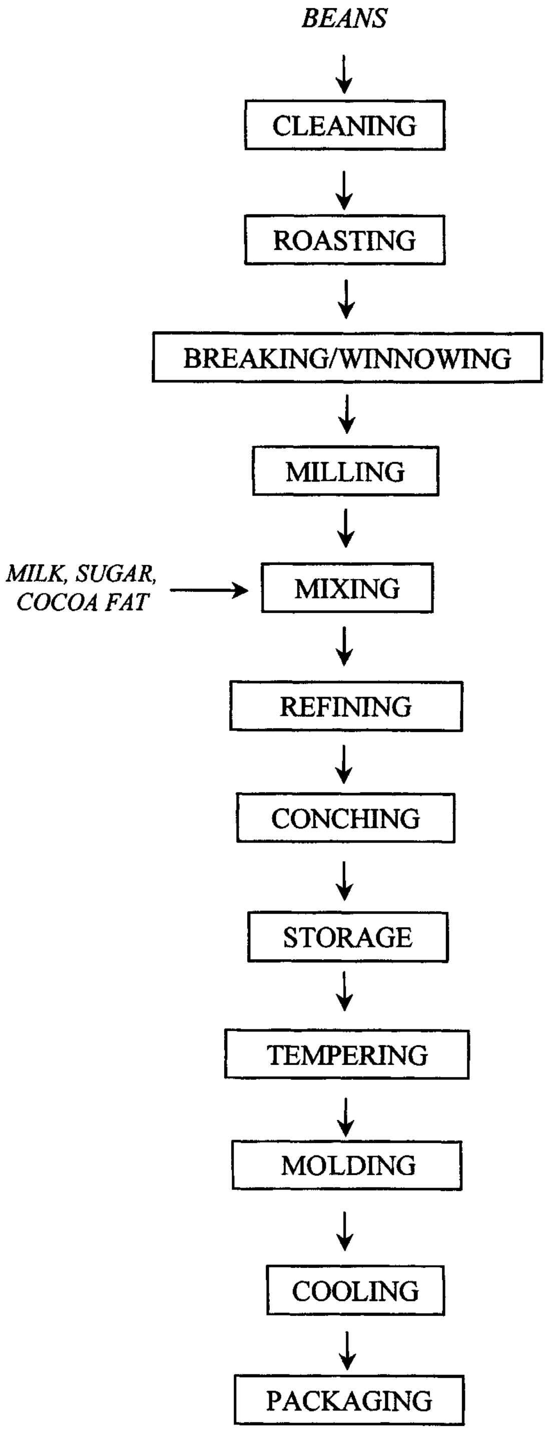

Figure 35-4 Flow Diagram of Chocolate Production.

such as salmonellae. Other processes, such as infrared heating or steam treatment designed to enhance separation of shells from beans and to minimize fat losses, may even allow destruction of spores.[95] However, the most important reduction is normally achieved through elimination of shells during winnowing.[86,92] As can be expected, the microflora of roasted beans is composed of spores, and species such as *Bacillus stearothermophilus, B. licheniformis, B. subtilis, B. coagulans, B. circulans, B. brevis*, and *B. megaterium* have been reported.[5,112]

For the production of chocolate, roasted beans, nibs, or liquor are submitted to several processing steps: milling and refining to obtain the desired granulometry; mixing to blend the cocoa mass evenly with ingredients such as milk powder, sugar, and cocoa butter; conching to agitate the chocolate mass in order to achieve the required degree of plasticity and allow for flavor development; tempering during which the fluid chocolate is submitted to temperature cycles to favor correct fat crystallization; and finally molding.[94] These steps have no major effect on the original flora as shown by analysis of finished products.[25] The levels of microorganisms depend on the initial flora and the roasting conditions applied. Minor changes in the species found may be influenced by added milk powder, sugar, or other ingredients, such as nuts or dry fruits added before molding. The presence of heat-sensitive microorganisms, including indicator organisms or salmonellae, is indicative of postprocess recontamination from added ingredients or from the environment.

In the case of cocoa powder, either nibs, cocoa liquor, cocoa powder, or press cake is exposed to alkali and temperatures of 85° to 115°C (alkalinization or dutching). The physicochemical changes in flavor and color achieved under these conditions are summarized by Minifie[94] and bactericidal effects also occur. Contamination by microorganisms such as spore formers[43,99] during the subsequent processing steps (pressing to extract cocoa butter, breaking of the press cakes, grinding of the kibbles, cooling, and packaging of the powder) is normally due to dust from the environment and can be minimized by protecting the product.[94] The heat generated during grinding may lead to condensation and thus to the development of molds in ducts and conveyors if no appropriate measures are taken to dry cooling air.[94] Viable counts in excess of 10^4 cfu g^{-1} indicate poor manufacturing practices.[24,90]

A very wide range of products is included in the categories chocolate confectioneries and sugar confectioneries, ie, bars, blocks and bonbons, boiled sweets, toffees, fudge, fondants, jellies, pastilles, and many others including very regional or local products. Manufacturing processes for several of these products are described by Minifie.[94] Processing conditions, in particular heat treatments, may vary greatly depending on the characteristics of the product manufactured. Treatments may range from very mild warming with limited killing effect to very effective cooking or boiling achieving complete destruction of vegetative organisms.[129]

35.5.4 Pathogens and Spoilage Associations

Although survival of *Staphylococcus aureus* in chocolate has been described, growth is not possible and therefore enterotoxin synthesis can be excluded.[110] *Salmonella* is the only bacterium that has caused outbreaks of disease associated with cocoa or chocolate.[29,68] Several episodes of salmonellosis have occurred over the last 20 to 25 years (summarized in Table 35-1). Some of these outbreaks have had an important economic impact, in some cases causing collapse of the companies involved.[120,141] A recent survey of chocolate in Mexico has shown that the problem is still of concern, even if no further outbreaks have been reported.[144]

In the case of chocolate, infections are due to the conjunction of very particular conditions. *Salmonella* has been shown to survive over very extended periods of time, even years in naturally contaminated products.[119,132,133] The pathogen also shows a strongly increased heat resistance because of the low water activity of chocolate and the protective effect of fat. Temperatures of 70°–80°C reached during milling, refining, or conching and even after overheating at >100°C were not sufficient to destroy small numbers of *Salmonella*.[53,54,119] A killing effect at around 70°C was achieved in some trials only after addition of 2% of water.[4] Application of this procedure in practice would, however, require proper validation as recommended by the IOCCC.[68]

In several of the outbreaks associated with chocolate, low levels of contamination have been reported (Table 35-1). On the basis of the quantity of product consumed or the fraction of the product contaminated (eg, only in the case of confectionery), very low infective doses, ≅10 to a maximum of 100 salmonellae, have been estimated. These remarkably low infective doses may be explained by the short intragastric residence of chocolate and the protective effect of fat allowing pathogens to enter the intestinal tract.[29,133] Chocolate and confectionery products do not support growth of pathogens. However, if contamination with *Salmonella* occurs, then the pathogen can survive for prolonged periods of time.[32,46,54]

Chocolate is characterized by very low water activities ranging between 0.4 and 0.5[118] thus impeding any spoilage. One single publication representing a special situation dealt with spoilage of chocolate containing different types of sweeteners and with varying equilibrium relative humidities (32% to 45%).[105] In this case, growth of molds at the interface between chocolate and packaging material has been attributed to the hygroscopicity of the sweeteners.

For cocoa powder, formation of off-flavors by molds metabolizing trichloranisol present in packaging material has been described.[150] Development of molds, however, is only possible if conditions are favorable as in the case of moisture uptake.

Spoilage of confectionery products depends very much on the water activity of individual products or product groups. The lower the water activity the more stable the product.[118] Thus, hard candy (a_w 0.2 to 0.35) is hardly affected, while jellies, marshmallow, or fondant (a_w 0.60 to 0.70) may suffer from spoilage due to osmophilic yeasts or xerophilic molds. Growth of fungi leads to visual deterioration of products such as marzipan, syrups, jams, candied fruits, confectionery, etc, by gas formation, fractures or bursting, formation of slime, color changes, development of off-odors and off-flavors, and enzymatic liquefaction.[13,20,71,93,98,143,151]

Table 35-1 *Salmonella* Outbreaks Linked with Chocolate Products

Year	*Outbreak*	*Serotype*	*Number of Cases*	*Level of Contamination [cfu g^{-1}]*	*Product*	*Origin of Contamination*	*Reference*
1970	Sweden	*S. durham*	110	?	Confectionery	Cocoa powder	44
1973	US/Canada	*S. eastbourne*	200	0.2–1.0	Candy	Environment	28, 30, 31
1982/1983	UK	*S. napoli*	245	2–24	Chocolate bars	Water	47, 56
1986	Canada	*S. nima*	29	0.005–0.025	Chocolate coins	Cross-contamination (?)	64
1986	Norway/Finland	*S. typhimurium*	>300	?	Chocolate	Birds	72, 73, 74

35.5.5 Preservation and Prevention of Contamination

The stability of chocolate is ensured by its low water activity, preventing development of spore formers as well as of contaminating organisms, including *Salmonella*. Thus, no further preservatives are required and safety of the products must be ensured by the application of appropriate preventive measures throughout manufacturing.

As mentioned above, roasting or alkalinization represents the only processing steps allowing destruction of *Salmonella* (critical control point, CCP). Because no further operation during production of cocoa powder, chocolate, and part of the confectionery products allows the destruction of vegetative organisms, recontamination with the pathogen must therefore be avoided by the introduction of appropriate measures based on the Hazard Analysis Critical Control Points (HACCP).

This starts at the level of the raw materials. Raw materials of agricultural origin such as raw milk, raw nuts, or raw fermented beans are not CCPs and control must be exercised by carefully monitored heat treatments. Processed raw materials can be subdivided into high risk (eg, gelatine, starch, egg products, dairy products) and low risk (eg, sugar, cereals) and preventive measures adapted accordingly. Preventive tools range from the careful choice of suppliers, the performance of audits, the verification by means of analyses for *Salmonella* and for indicators, such as coliforms, and examination of recorded monitoring data. Rework plays an important role in the chocolate/confectionery industry, and it should be handled as high-risk raw material. Further raw materials to consider are water, used in large quantities in jacketed systems, and air, which may be introduced directly into the product.

In processing plants, particular attention must be paid to the layout of buildings, premises, equipment, and personnel movements to allow for an optimal segregation between clean and unclean, and wet and dry zones. Extensive discussions on HACCP for cocoa, chocolate, and confectionery products can be found in different publications.[26,67,68] Guidelines of Good Manufacturing Practices (GMP) for the same group of products have been issued by the IOCCC.[69]

The several outbreaks presented in Table 35-1 underline the failure of preventive measures and were attributed to contaminated raw materials in the *Salmonella durham* outbreak,[44] inadequate layout and cross-contamination in the *S. eastbourne* outbreak,[28] probable contaminated water in the *S. napoli* case,[47] and probable cross-contamination through birds in the most recent *S. typhimurium* outbreak.[73,74] For the cocoa powder also, no additional preservatives are necessary, the low water activity being sufficient to guarantee stability.

In the case of confectionery products, preservation is ensured using different hurdles depending on the characteristics of the products, these hurdles include heat treatments, low water activities, or organic acids (acidulants). Contamination and development of spoilage fungi introduced with ingredients such as flours or starches, sugars or syrups, nuts, dried fruits, or jams or from the environment and air can be minimized or avoided by preventive measures, strict adherence to hygiene and good manufacturing practices and maintenance of the effective hurdle. Failures may lead to contamination and subsequent spoilage by osmophilic yeasts or xerophilic molds.[35,148]

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36 Teas, Herbal Teas, and Coffee

Martin J.M. Michels

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References

36.1 INTRODUCTION

The products in this chapter have in common that they are prepared with boiling water and consumed as a drink. They are products of long standing of which tea appears to be the oldest, with a history of 2,000 years.[93] In the Western world, tea and coffee drinking became a habit about 300 years ago but herbal infusions have been used as a beverage and for medicinal purposes since the Middle Ages. The world annual production of tea is 3 million tons and that of coffee nearly 6 million tons; herbal teas form only a small fraction of the coffee and tea market.

36.2 TEA

36.2.1 Composition

Tea is produced in the tropical and subtropical regions of the world from the tea plant *Camellia sinensis*.[8] The plant is grown as a bush at high elevations (typically 500–2,000 m); the young top leaves of the bush are plucked and used for making black or green tea. The basic difference between these teas is the way the leaves are processed. Green tea is unfermented and the leaves are "fired" directly after picking to inactivate the leaf enzymes that would otherwise initiate oxidative fermentation. Black tea is made by initiating an enzymic oxidative fermentation process between picking and "firing." The fermentation of the leaf is caused by enzymes released from the tea after withering (moisture loss) and maceration. Enzyme activity is stopped by the drying process ("firing") thereafter. A third type of tea (oolong) is a semifermented tea that is allowed to ferment for a short period only. Black tea forms *ca.* 75% of the total volume of tea produced with *ca.* 25% being green tea. Much of the green tea is consumed in China and Japan and is not exported.

Black tea is defined according to International Standards Organization (ISO) 3720 as "Tea derived solely and exclusively, and produced by acceptable processes, notably fermentation and drying, from leaves, buds and tender stems of varieties of the species *Camellia sinensis* (Linneaus) O. Kuntze, known to be suitable for making tea for consumption as a beverage."[42] According to this standard, tea must have no taint and must be reasonably free from extraneous matter. Other chemical requirements are set to prevent the adulteration of tea with minerals, the sale of spent leaf, and to ensure the adoption of good plucking and sorting standards (a minimum of 32% water-extractable solids, a maximum of 16.5% crude fiber, a maximum of 1% acid-insoluble ash, and a total ash requirement of 4% to 8%). The moisture content, which may be determined by drying at 103°C, is not specified in the ISO standard, although it is the low moisture of the final product that ensures the long storage life of tea. In practice, the moisture content of high-grown black tea may range from 3% to 4% directly after drying, to about 7% to 8% after shipment to the tea packing plant. Low-grown tea (cultivated below 500 m altitude) is exposed to more humid conditions than tea grown at higher altitude and may have moisture levels close to 10% (see Section 36.2.3). For green, artisanal tea this may be above 10%.

The main characterizing components of tea are its polyphenols, often incorrectly referred to as tannins. The main polyphenols of green tea (the colorless catechins) are converted during the fermentation step of black tea production into the red-brown theaflavins and thearubigins. The polyphenols of black tea constitute 10% to 20% of its dry weight, and *ca.* 30% of the solids extracted during brewing of the tea to prepare as a beverage. The other important characterizing component of tea is caffeine, which constitutes 1.5% to 5% of its dry weight and 5% to 8% of the solids extracted during brewing.

36.2.2 Initial Microflora

Little has been reported about the microbial load of fresh tea leaves. Analysis of fresh leaves of the Yabutika cultivar, commonly used for green tea production in Japan, established a mold and yeast count of 7,500 g^{-1} of which *ca.* 2,500 g^{-1} was tentatively identified as *Cladosporium cladosporoides*.[15] Fresh leaves from gardens near Bangalore (India) were analyzed (after frozen storage) on three separate occasions (in March, May, and July). The counts obtained varied from 14,000 g^{-1} (May) to 193,000 g^{-1} (July), with an intermediate count of 82,000 g^{-1} in March. Mold counts ranged from 45 g^{-1} in July to 600 g^{-1} in March. Yeasts were not detected ($<10\ g^{-1}$).[53]

36.2.3 Effect of Processing

Green Tea (Unfermented)

In Japan, tea is plucked for steamed, green tea over three seasons of the year. The plucked leaves are first steamed for 45 to 60 seconds to inactivate the leaf enzymes, and then curled and dried in hot air at 90°–100°C for 40 to 45 minutes. During this first drying step, the moisture content is reduced from 76% to *ca.* 50%. This is followed by two successive drying and rolling steps, which are concluded with a final drying process at 80°C, to a moisture content of 6%. Pan-fried Chinese green tea is manufactured by firing fresh leaves in a pan at 250°–300°C for 10 to 15 minutes with frequent agitation to prevent burning of the leaves. The leaves are finally rolled and dried at 100°–150°C in a pan.[81] The steaming or pan-firing of the leaves will not only inactivate enzymes, but may also kill most microorganisms and result in a low count. This is in line with a mold count of 250 g^{-1} for a sample of Japanese green tea and of 750 g^{-1} for a sample of 100-year-old green tea.[15]

Oolong Tea (Semifermented)

Fresh tea shoots are withered (to reduce moisture content) under sunlight for 30 to 60 minutes, followed by 6 to 8 hours indoor withering on a floor, with gentle hand rolling and agitation. The moisture content is reduced from 78% to 60% during this operation. After this controlled withering process, the leaves are "fired" in a pan at 250° to 300°C for 15 minutes to stop the fermentation. This is followed by further rolling and drying as for the pan-fired green tea.[81] As noted for green tea, the pan-firing will inactivate most microorganisms and the dry tea will have a low microbial load as a result. A mold count of 50 g^{-1} was obtained for a sample of oolong tea.[15]

Black Tea (Fermented)

Black tea manufacture comprises essentially five steps.[35] These are: (1) partial removal of moisture from leaves (withering); (2) leaf disintegration into small pieces (cutting, rolling); (3) quality development by exposure to air (oxidation or fermentation); (4) completion of moisture removal (drying or firing); and (5) sieving into size fractions with fiber removal (sorting). Step two is done by (i) the orthodox process, (ii) the CTC process (crush, tear, curl), or (iii) the LTP process (Lawrie Tea Processor).[35,92]

Withering results in a significant increase in bacterial count (by a factor of 5 to 50). For instance, counts were 0.5 to 2 million after withering of the previously mentioned Indian teas (Section 36.2.2); the mold counts remained relatively low (1,000 to 10,000 g^{-1}) and only a few yeasts were found.[53] Following the rolling and cutting process, there was little change in counts.

During fermentation, cytoplasmic polyphenol oxidase reacts with the polyphenols from the disrupted cell vacuole. The latter process produces heat with the uptake of oxygen and the fermentation results in the formation of the pigmented, water-soluble polyphenolic compounds typical of black tea.[71] The fermentation may take between 40 minutes and 3 hours depending on temperature and process used. Traditionally, the fermentation took place in a thin layer of tea leaves on the floor, which ensured adequate air supply, and good cooling of the leaves, as fermentation is best carried out at 30°C. This was followed by fan-assisted deep layer fermentation in which air was blown through tea leaves via a perforated floor. In modern continuous fermentation, the "deep bed nonperforated system," the tea leaves are held in a large installation in thick layers, which are regularly exposed to air, to provide a hygienic and temperature-controlled fermentation during which the leaf temperature can be well controlled.[35] The main result of the fermentation is the production of the theaflavins and thearubigins that characterize black tea. During fermentation, the bacterial counts increased slightly to 1 to 10 million in the Indian teas studied by Marthi[53]; the mold counts remained unchanged (*ca.* 1,000 g^{-1}) but the yeasts started to grow reaching levels of 50,000 to 500,000 g^{-1}. In another study[69] bacterial and fungal counts apparently decreased slightly (by a factor of 2 to 3) during the first hour of fermentation but this may be within the statistical variation of the analytical method. As with the production of any food product, proper control of hygiene, and in particular the cleaning/sanitation of the equipment and surfaces in contact with the tea, is important to prevent the build-up of high concentrations of microorganisms, leading to taints and other undesirable quality defects.[69,92]

The microbial flora may also contribute to the fermentation process by their polyphenol oxidase and peroxidase activity. Out of 10 bacterial isolates from tea, 8 showed polyphenol oxidase activity, the most active being three *Bacillus* spp., which also showed peroxidase activity.[68]

The microbial load is significantly reduced during firing. The rate and amount of inactivation depend on the profile of the dryer. The temperature of the leaf during drying is not equivalent to the temperature of the dryer because of evaporative cooling of the material being dried. In trials in India, tea fired at a temperature of 135°C resulted in a maximum leaf temperature of *ca.* 90°C. This would be expected to give a significant decrease in count but this was not always observed. Thus, two samples of "fired" tea had counts of 5,000 and 20,000 g^{-1}, whereas a further sample had a count of 6 million g^{-1}. Mold counts were low (55 g^{-1} to 3,550 g^{-1}) and yeast counts varied from 3,000 to 40,000 g^{-1}.[53] The high temperatures are only reached at the end of the firing process, at a time when the heat resistance of surviving microorganisms will be increased because of the low water activity of the leaf at the end of the drying process. In countries with high humidity (low-grown teas), air drying is intrinsically more difficult and teas tend to have moisture contents above the target of 3% to 4%, although also drier design factors (overloading, delays in packing, inadequate capacity) may be a contributor.

After the main processing steps, the tea is graded and sorted. During this sorting operation, it is important that humidity is controlled as dried tea easily takes up moisture from the surrounding air.[35,92] After grading, the tea is bulk packed for transportation to the tea auction. Traditionally, wooden chests were used, but nowadays most fannings, dust, and broken grades of tea are packed in multiply Kraft paper sacks with a polyethylene/aluminium barrier liner that better protects the tea from the ingress of moisture.

From the auction, the tea is transported to the tea packing plant, where the tea is unpacked, sieved to eliminate stalks and other foreign bodies, and then blended in large rotating drums to produce the tea quality required by the customer. At this point in the production chain, black tea will have a typical moisture content of 4% to 7% (maximum, 10%).

Decaffeinated Tea

The caffeine present in tea can be extracted by methylene chloride, ethyl acetate, or super-critical carbon dioxide. As caffeine inhibits aflatoxin formation (Section 36.4.3), its removal may make decaffeinated tea more likely to support aflatoxin production should it become wet and moldy.

Instant Tea

About 1% of tea is transformed into instant tea. The tea raw material may be black tea (fermented undried leaf) or oolong or green tea. The tea is extracted with water at temperatures from 15°–100°C in a batch or continuous process. The yield of soluble solids is *ca.* 25% to 35% of the weight of the dry leaf. The tea extract or liquor is normally treated with additional processes before it is concentrated and dried.[74]

Concentration of the tea liquor is by evaporation, reverse osmosis, or freeze concentration until a liquor is produced with a solids content of *ca.* 35%. The final step in the process is spray drying the liquor. Vanos et al.[91] studied the microorganisms present at different points in the instant tea production process, as well as the effect of tea components on pathogens and spoilage microorganisms. Lactic acid bacteria were predominantly present, with *Lactobacillus plantarum* the major species. To prevent the growth of lactobacilli, the process line should be kept at <10°C or >50°C. The pathogens studied were unable to grow in the tea liquor.

36.2.4 Spoilage Associations and Pathogen Hazards

Spoilage

Tea processing includes a number of steps where microbial numbers may increase or decrease as described previously. The initial drying to 3% to 4% moisture ensures that no microbial growth can occur in the dried black tea. Such tea will take up moisture from the environment to give a moisture content of 5% to 7%; the moisture content of green teas and low-grown black tea may increase more to 10% to 11%. This will not, however, result in spoilage as moisture levels close to 15% are required for spoilage to occur. With respect to spoilage, the concern is mainly the possible growth of molds, when tea becomes sufficiently wet to reach a water activity of 0.7. This may occur during transportation and storage and is normally visible as a recognized defect. Tea chests and sacks containing moldy tea should not be used.

Pathogen Hazards

There is no substantial evidence for mycotoxin formation in moldy tea except for a report of the finding of 2.8 to 18.4 µg aflatoxin B_1 and B_2 kg^{-1} in four samples of Egyptian tea powder, heavily contaminated with *Aspergillus flavus* and *A. parasiticus*[1]; the tea when tested had a normal moisture content of 5.8 to 8.1%. When wetted and inoculated with *A. flavus*, 72 µg aflatoxin B_1 and B_2 kg^{-1} were produced after 2 weeks storage at 28°C followed by 2 weeks at 15°C.

The European Union's Scientific Committee on Food recently reviewed the microbiological risks associated with tea and concluded that: "Tea has a long history of safe use and the Committee is unaware of any safety problems related to moisture in tea. This may be attributed to its low moisture content (ie, water activity) and the high content of antimicrobial substances. Moisture levels up to 10% seem to give an acceptable safety margin for the storage of tea. A lower level may be needed to restrict quality defects."[77]

36.2.5 Antimicrobial Activity

Laboratory tests have demonstrated that tea has significant antimicrobial properties. Thus, Ryu demonstrated that 0.5% tea powder inhibited *Staphylococcus aureus, Vibrio cholerae, Salmonella typhi,* and *S. paratyphi* but that *Escherichia coli* was only slightly inhibited; a 3% suspension killed *V. cholerae,* with an initial concentration of *ca.* 100 to 1,000 bacteria ml^{-1}, in 30 minutes.[72] Further studies with 1% oolong tea powder extended inhibition by tea to *S. typhimurium, Proteus mirabilis,* and *Pseudomonas aeruginosa; Klebsiella pneumonia* was resistant.[73] Ryu hypothesized that tea tannins may be responsible for the antimicrobial activity, and it was later confirmed that tea polyphenols (catechins and theaflavin) were responsible for growth inhibition of *Clostridium botulinum* and a number of other Gram-positive and Gram-negative pathogens including *Vibrio parahaemolyticus, V. fluvialis, V. metchnikovii, S. aureus,* and *C. perfringens.*[36,37] The latter study found no activity of tea polyphenols against *E. coli, Yersinia enterocolitica* or *Campylobacter jejuni,* and *C. coli.* There are thus significant differences in the sensitivity of bacterial species to inhibition by tea and tea extracts. A strong decoction of tea (25 to 30 g in 150 ml water) is often used as therapy in India during an episode of diarrhea,[78] whereas Japanese green tea is bactericidal to a number of pathogens at a concentration of tea used in daily life.[88] A recent mini-review gave a comprehensive literature survey of the antimicrobial properties of tea.[34]

36.2.6 Preservation

There are few published data on the microbial flora of packed teas. Zhao et al.[96] analyzed 25 samples of leaf tea for coliforms and fecal coliforms and reported that the numbers of coliforms and fecal coliforms were <10 g^{-1} in 32% of the samples, between 10 and 100 g^{-1} in 60% of the samples, and above 100 (maximum 1,100) in 8%. Data recently collected from a few tea packing stations in India are shown in Table 36-1.[14] These data represent only tea from a limited source and may not be representative of different teas from different sources. For instance, green tea from China may have up to 10 times higher counts because of the more artisanal firing process applied. Spain is one of the few countries with mi-

Table 36-1 Aerobic Plate Count, Yeasts/Molds and Coliforms of Indian Retail Black Tea ($n = 182$)

Counts g^{-1}	*0–*10^3	*>*10^3*–*10^4	*>*10^4*–*10^5	
Aerobic plate count	28%	65%	7%	
Yeasts and molds	45%	53%	2%	
Counts g^{-1}	*0–10*	*>10–*10^2	*>*10^2*–*10^3	*>*10^3
Coliforms	65%	18%	14%	3%

crobiological limits for tea: aerobic plate count max. 1 million g^{-1}, *Bacillus cereus* max. 10^3 g^{-1}, *E. coli* max. 10 g^{-1}, molds max. 10^4 g^{-1}, and absence of *Salmonella/Shigella* in 25 g.[4]

The excellent preservation status of dried tea comes to an end when tea is infused with boiling water to prepare a cup of tea. When tea is consumed hot, there will be no microbial problems. In fact the normal brewing of tea will inactivate the few non–spore-forming organisms present and a perfectly safe drink will result. This holds also for iced tea, when first a hot tea is prepared with boiling water and the drink is chilled thereafter with ice cubes or in a refrigerator.

However, under abuse preparation and holding conditions, bacterial growth may take place in brewed and iced tea. There have been instructions on the label of packed black tea in the United States (US) to prepare iced tea with cold water by brewing the tea in the refrigerator for a few hours. Such instructions can lead to contamination with coliforms and other organisms present in tea. Claimed high levels of fecal coliforms in iced tea drinks have caused concern to the authorities in the US and comment in the media. However, these so-called "fecal coliforms" were in fact *Klebsiella* and *Enterobacter* species, organisms that are common in tea; in fact no *E. coli* was present.[24] It has been pointed out that species of *Klebsiella* and *Enterobacter* are commonly associated with plants and plant materials, where they do not represent fecal contamination. The "fecal coliform" levels in iced tea samples from restaurants in the US varied from 15 ml^{-1} to 1,100 ml^{-1} and high numbers were the result of poor hygienic practices in the preparation and storage of the iced tea.[96]

It is not recommended that tea be prepared by brewing in the sun (sun tea) as is suggested on the label of some teas sold in America. Sun brewing for a few hours will result in temperatures that are ideal for microbial growth, and organisms such as coliforms, that will often be present in low numbers, will start to grow and reach numbers that are no longer in line with good hygienic practice. It is therefore recommended that proper instructions (text or pictogram) are given on packs of dried tea to use boiling water to prepare a tea drink and to use water over 85°C for tea in vending machines. After water, tea is the most common drink in the world. Tea is also probably the safest beverage in the world.

36.3 HERBAL TEAS

36.3.1 Composition

Herbal teas are not, formally speaking, teas as they are not made from *Camellia sinensis;* they are often referred to as herbal infusions or as infusions.[94] They are the dried aromatic parts of plants consisting of root, root-stock, shoots, leaves, flowers, barks, fruits, or seeds, which after preparation with boiling water are suitable as a drink. A few examples are chamomile (flower), mint (leaf), licorice (root), rosehip (seed), and apple (fruit). A list of over several hundred herbal teas and drugs is given by Hörhammer[41] but the number used commercially for herbal tea production is well below 50.

Herbal tea raw materials are cultivated in many countries of the world as they are grown where climatic conditions are most suitable for particular plants. Thus, chamomile is grown in Italy and Latin America, hibiscus in Morocco, rooibos in South Africa, and mint in many European and African countries. Some of these, such as mint, are harvested as moist materials and dried thereafter, while others, such as chamomile flowers, are dry when collected.

36.3.2 Initial Microflora

Few studies have been done of the microbial flora of herbal tea materials in the field. The flora on lavender (*Lavandula angustifolia*) and nine leaf plants growing in a garden in the University of Messina (Italy) was investigated.[2] The lavender flowers had an aerobic plate count (APC) of *ca.* 2.5 million g^{-1} (comprising mainly yeasts) whereas the leaf materials had lower counts of 10^4 g^{-1} to 5×10^5 g^{-1} and comprised variable numbers of Gram-negative and spore-forming bacteria. Chamomile and peppermint grown by three French growers were analyzed.[10] The average APCs on fresh materials from each of the three growers of chamomile were 2.5×10^5 g^{-1}, 2.9×10^6 g^{-1}, and 1.0×10^8 g^{-1}, ie, there was nearly a thousand-fold difference in microbial numbers on chamomile samples from the three growers. Numbers of yeasts and molds on the fresh chamomile were 10^4 to 10^5 g^{-1}. Bacterial groups present included fecal streptococci, pseudomonads, Enterobacteriaceae, and sulfite-reducing clostridia. The APC results for the fresh peppermint showed similar large differences in the levels of microbial contamination on materials from different growers.

36.3.3 Effect of Processing

Little has been published on the effects of processing (mainly drying) on the microbiology of herbal tea raw materials. However, it is apparent that the initial microbial load will be high and the types of microorganisms, as would be expected on any raw plant material, will be very variable. Mild conditions are used for drying to avoid loss of flavor, color, or essential oils, and such conditions do not have a marked effect on the microbial flora.[2,10,56] For balmmint, the dried material had counts of *ca.* 10^5 g^{-1}, quite similar to the fresh and semiprocessed raw material.[76]

The production of rooibos tea, prepared from an indigenous South African shrub (*Asphalathus linearis*) is different from that of other herbal teas because it is the only one that is fermented. Young green twigs with needle-like leaves are collected from plantations, transported in bundles to processing farms where they are cut into pieces of *ca.* 5 mm in length. Water is added, and the twigs are bruised and put into heaps to ferment for 8 to 24 hours at about 30°C (24° to 38°C). During the fermentation, chemical changes take place as a result of the enzymes released from the leaves and their color turns to amber-red. Microbial numbers increase during the fermentation and prefermentation handling stages. For example, in one study coliform bacteria increased from 200 g^{-1} on material prior to harvest to 3.6 million g^{-1} after fermentation and APCs increased from 3×10^4 g^{-1} to 4×10^8 g^{-1}.[25] After fermentation, the heaps are turned over and the rooibos tea is dried for 1 to 2 days in the sun, sorted and graded and finally pasteurized with steam, followed by hot air drying (Section 36.3.5).

36.3.4 Spoilage Associations and Pathogen Hazards

Spoilage

Although many herbal infusions have high microbial counts, spoilage is rare as they typically have a low moisture content of 7% to 12% corresponding to a water activity of 0.4 to 0.6.

There are many reports on the microbial load on herbal infusions used for medicinal use, with the general and not unexpected conclusion that the load is high and variable, but there are few comparable data for herbal teas consumed as a beverage.[65] The European Herbal Infusions Association (EHIA) therefore collected microbiological data of *ca.* 1,000 samples of herbal teas from all parts of the world. The teas examined had not been fumigated. The data obtained for APC are shown in Figure 36-1 and represent the main varieties (chamomile, linden, mint, orange leaves, and verbena) and also minor varieties of teas; data for molds are also presented in Figure 36-1. For yeasts, numbers ranged from 10^2 to 10^7 g^{-1}, with counts on mint in excess of one million g^{-1} (data not shown).

Pathogen Hazards

Salmonellas are generally not found in herbal drugs or teas.[3,44,46,47,65,75,95] However, Frank[30] refers to a survey of 374 samples of herbal tea in which 1.3% were contaminated with salmonellas. Bockemühl[12] reported finding *S. schwarzengrund* in a sample of peppermint, and Eckner et al.[26] reported salmonella in 4 out of 10 samples of lemon verbena (MPN 0.004 g^{-1}). The general low incidence of salmonellas in herbal tea is in line with the findings of Unilever Research.[57] Some strains isolated were *Salmonella montevideo* and *S. london* from linden tea, *S. weston* from verbena, *S. typhimurium* from mint, and *Salmonella* (not typed) from rooibos tea.

The incidence of salmonella in rooibos tea, however, has been reported to be high with 20% to 100% of South African rooibos samples contaminated, some with multiple serovars.[66] The exact source of the contamination recorded in this study was not identified. However, contaminated dust, human handlers, contaminated water used in the fermentation stage, and excreta from insects, birds, or animals are all possible sources. In one particular study, more than 150 salmonellas (49 serovars) were isolated from rooibos over a period of 1 year. The predominant serovars were subspecies of *S. choleraesuis (S. choleraesuis salanae and S. choleraesuis arizonae)*, and it is suggested that lizards were the main source of contamination.[80] However, other serotypes isolated from rooibos tea suggest other sources of contamination including water and other animals.[66] The high prevalence of salmonella on rooibos tea led to the introduction of a steam decontamination process (Section 36.3.5).

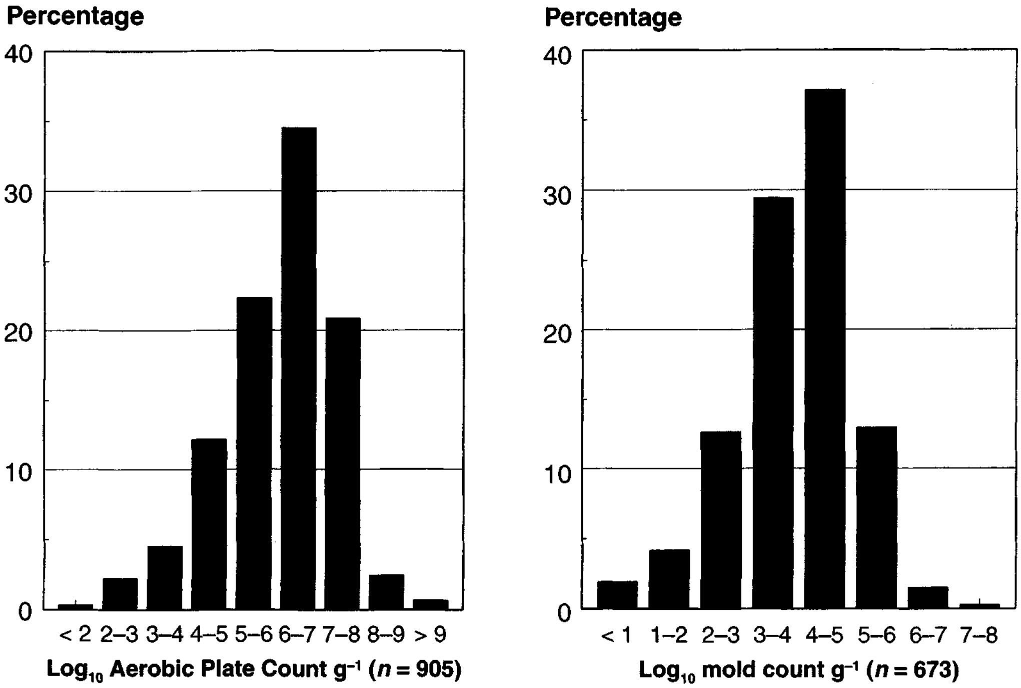

Figure 36-1 Aerobic Plate Count and Mold Counts of Nonfumigated Herbal Teas.

Other pathogens found contaminating herbal teas include *Bacillus cereus* (most teas have only a low load but chamomile often has a high load, typically 10^4–10^5 g^{-1}) and *C. perfringens*, which is present at low levels (10–1000 g^{-1}) in about 50% of teas, and occasionally at higher levels; bacterial spore-formers comprise 10% to 90% of the microbial flora of herbal teas. *Escherichia coli* may also be found and in the earlier mentioned EHIA survey most levels were low (73% below 100 g^{-1}) but 25% of samples had counts of 10^2 to 10^4 g^{-1} and a few above 10^4 g^{-1}. A low prevalence of *Staphylococcus aureus* was also reported.[30]

Herbal drugs and teas have been extensively examined for the presence of mycotoxins and, in particular, aflatoxins. Lutomski and Kędzia[50] examined 246 samples of 95 herbal drugs for aflatoxin B_1, ochratoxin A, and sterigmatocistin and failed to find these mycotoxins. These mycotoxins were not found in a Japanese study of 49 herbal drugs.[40] Aflatoxins were not found in other studies of herbal teas.[39,46]

36.3.5 Preservation

Because of their low moisture content, herbal teas are well preserved. However, in view of their often high microbial load, and the possible presence of pathogenic microorganisms, the effect of procedures used to prepare these teas for consumption has been studied extensively.[10,23,33,38,46,65,85,89,95] Boiling water was used in most of these investigations but water at 80°C was included in studies at Unilever Research. Filter bags of chamomile and mint with a natural contamination of 1 million Enterobacteriaceae g^{-1} were infused for 5 minutes and the resultant drink (and also the filter bag) analyzed for Enterobacteriaceae; no survival was observed.[57]

The conclusion from these studies is that herbal infusions when properly prepared, ie, by using boiling water, are microbiologically safe. Attention must therefore be given to providing clear instructions on packs like "Prepare with boiling water"; or a set of pictograms giving the same message. Instructions on some medicinal teas, which refer to the use of "hot water," will not ensure safety and should not be used.

Once prepared, infusions should be consumed shortly thereafter because surviving spore-forming bacteria may grow in the prepared drink when stored for over 24 hours at ambient or higher temperatures.[23,46,95] *Bacillus cereus* multiplied from 66 cells ml^{-1} to 2.5×10^5 ml^{-1} in a properly prepared chamomile tea stored for 24 hours at 30°C; no growth was observed in infusions of mint and linden prepared and stored under the same conditions.[57]

To illustrate the need for proper and suitable instructions for preparing herbal teas, an instruction for a medicinal drug (*Harpagophytum procumbens* DC 1) is presented below to illustrate an instruction that is **not suitable** for herbal teas:

> "Devils Claw tea should be prepared in the evening and allowed to stand overnight so that it may brew and develop its full effects. Place the filter bag in a glass or porcelain pot and add ¾ pint of boiling water. Remove the filter bag next morning. Drink the tea, thus obtained, either cold or lukewarm in three equal portions, 10 minutes before morning, noon, and evening meals. Do not boil the tea."

Decontamination of Herbal Infusions. Ethylene oxide (EO) has been widely used to decontaminate the materials used in herbal teas. However, its use was banned in the European Union (EU) in 1990 because of concerns over the occupational safety hazards associated with its use. There were also some concerns over the presence of EO residues and the concomitant production of the mutagen ethylene chlorohydrin (ECH) in the fumigated material.[33,75] EO is still allowed to be used in some countries. For example, it is permitted in North America with the consumer hazards controlled by setting limits for residual levels of EO (50 mg kg^{-1}, US) or ECH (1,500 mg kg^{-1}, Canada).

An effective alternative to EO decontamination is irradiation.[21] Irradiation with ^{60}Co gamma rays at level of 5 to 10 kGy commercially sterilized chamomile flowers without loss of quality or chemical changes.[44,45] In South Africa, irradiation of rooibos tea is officially permitted and was used to decontaminate rooibos in the 1980s.[66] Irradiation studies of six varieties of herbal teas by the EHIA showed that a dose of 10–15 kGy was required to eliminate Enterobacteriaceae and that there was no effect of this treatment on the acceptability of the infusions.[32] Chile is another country where irradiation of herbal teas currently is permitted and used. Extrusion decontamination is also potentially applicable to herbal teas. Although the process is effective in killing microbial contaminants, the appearance of the infusions is affected.[67]

Steam treatment is the method of choice for decontaminating rooibos tea. In initial experiments to establish a suitable process, rooibos with a moisture content of 10%, 15%, and 20% was heated as a 5 mm layer in a steam cabinet at 99.5°C for 2 to 5 minutes. This reduced the level of coliforms from over 10^5 g^{-1} to <10 g^{-1}. The organoleptic quality was acceptable to the Rooibos Tea Board only for rooibos treated for 2 minutes at 10% moisture.[25] In a slightly different treatment (2 minutes at 92°–95°C, followed by drying at 36°–38°C) salmonella were not detected immediately after the heat treatment but were recovered from 60% of treated samples stored at 4°C for 33 weeks; salmonella were not detected in treated samples stored at ambient temperature or at 37°C.[70] Currently, all rooibos is pasteurized for 2 minutes at 90°C and meets a specification of salmonella absent in 25 g, *E. coli* maximum 20 g^{-1}, aerobic plate count maximum 50,000 g^{-1}, yeasts and molds maximum 5,000 g^{-1}.[9]

Microbiological Limits. A number of microbiological limits have been proposed and applied to medicinal and herbal teas (Table 36-2). Those of the EHIA[27] are based on a large database and used in Europe for the trade in herbal infusions.

36.4 COFFEE

36.4.1 Composition

Coffee is cultivated from a few varieties of the genus *Coffea* in tropical and subtropical areas of mainly Latin America, Africa, and India. The main varieties grown are *Coffea arabica* (*ca.* 75% of the world production) and *Coffea robusta,* a form of *Coffea canephora* (25% of world production).[43]

The coffee cherry or berry consists of an outer layer of pulp and an inner layer of mucilage in which two seeds (beans) are present. The mucilaginous layer is removed from the beans by natural drying to give "natural" coffee or by a wet fermentation process to give "washed" coffee. The purpose of fermentation is to remove the mucilage pulp around the beans and not the development of coffee flavor. "Washed" coffee is of superior quality to "natural" coffee.

36.4.2 Effect of Processing

For "natural" coffee production, the berries are ripened on the tree, harvested, slowly dried to 13% moisture or less, and the pulp layer and skin removed by hulling.[17] The "washed" coffee process consists of a number of steps: (1) classification (cleaning), by flotation of the berries; (2) pulping, to remove the skin and the pulpy layers around the beans; (3) fermentation, in large tanks; (4) drying; (5) hulling; and (6) grading/sorting.[17]

During fermentation, the pectin and pectic acid from the mucilage are broken down by a variety of

Table 36-2 Microbiological Limits for Herbal Drugs and Teas

Field of Application	*Aerobic Plate Count*	*Molds and Yeasts*	*Enterobacteriaceae*	*E. coli*	*Salmonella*	*Status*	*Ref.*
Pharmaceutical tea	10^3–10^4 g^{-1}	100 g^{-1}		absent in 1 g	absent in 1 g	proposed[a]	29
Herbal drug & herbal tea	10^6 g^{-1}			absent in 1 g	absent	proposed	10
Pharmaceutical tea	5×10^5 g^{-1}	5×10^3 g^{-1}	5×10^4 g^{-1}	absent in 1 g		proposed	47
Pharmaceutical tea	10^6 g^{-1}	500 g^{-1} for molds	500 g^{-1} coliforms	absent in 1 g		proposed	33
Pharmaceutical tea	5×10^6 g^{-1}	5×10^3 g^{-1}	5×10^3 g^{-1}	absent in 1 g	absent in 10 g	proposed	38
French Pharmacopoeia	10^6 g^{-1} (x5)[c]	10^5 g^{-1} (x5)				official	5
Pharmaceutical tea	10^8 g^{-1}	10^4 g^{-1}	10^7 g^{-1}	10^3 g^{-1}	absent in 10 g	proposed	30
German Pharmacopoeia	10^7 g^{-1} (x5)	10^4 g^{-1} (x5)	10^4 g^{-1}	100 g^{-1}	absent in 10 g	official	7
Pharmaceutical tea	5×10^8 g^{-1}	10^6 g^{-1} for molds				proposed	85
Italian Pharmacopoeia	10^7 g^{-1} (x5)	10^4 g^{-1} (x5)			absent in 10 g	official	6
Herbal teas	10^8 g^{-1}	10^6 g^{-1}		10^4 g^{-1}	absent in 125 g	industry[b]	27

[a] FIB = Fédération Internationale Pharmaceutique; joint report of the Committee of Official Laboratories and Drug Control Services and the Section of Industrial Pharmacists FIB.

[b] European Herbal Infusions Association.

[c] Factor 5, analytical tolerance permitted.

pectinases produced by molds, yeasts, and bacteria. Jones and Jones[43] give an extensive overview of the literature and refer to studies on Hawaiian coffee where Gram-negative bacteria such as *Klebsiella* species (formerly *Erwinia dissolvens*) were predominant; the same was true for African coffee berries. For Brazilian coffee berries, the main organisms were reported to be coliforms, resembling *Enterobacter* and *Escherichia.* Molds including *Penicillium, Fusarium, Cladosporium,* and *Aspergillus* were also found on Brazilian coffee berries. Pectinolytic yeasts (*Saccharomyces* and *Schizosaccharomyces)* formed the largest part of the microbial population on fermenting Indian robusta coffee.

Fermentation is carried out in large, deep tanks. The upper part provides aerobic conditions and the lower, anaerobic conditions, which are best for coffee quality.[43] The fermentation is rapid and the mucilage is degraded in 7 to 8 hours at ambient temperature, with fermentation typically complete in 24 hours. During fermentation, ethanol and acetic, lactic, propionic, and butyric acids are formed, and the pH is reduced from an initial pH of *ca.* 6.0 to 3.7. Prolonged fermentation of Colombian coffee cherries resulted in an increase in acetic acid (and later also formic acid), and in a vinegar-like taste after 68 to 110 hours and a "stinker" defect after 140 hours. Predominant organisms during overfermentation were lactic acid bacteria and anaerobic bacteria.[49]

After completion of fermentation, the residual mucilage is removed, fermentation is stopped by washing, and the coffee beans are dried. Drying to 10% to 11% moisture is achieved by sun drying (about 8 to 10 days) or by hot-air drying in a mechanical dryer or a combination of the two.[16] The dried beans are thereafter hulled to remove the parchment surrounding the green coffee beans. The graded and sorted beans are sold in sacks, bags, or containers. They are blended, roasted, and sold as roasted coffee, or ground and extracted to give instant coffee or instant decaffeinated coffee. They may also be sold as sterilized liquid coffee in cans. For instant coffee, the roasted and ground coffee is extracted with hot water in a series of percolation batteries and the concentrated extract spray or freeze dried. Details of these processes are described by Clarke and Macrae.[17]

36.4.3 Spoilage Associations and Pathogen Hazards

Spoilage

During extended fermentation, microbial degradation of the coffee beans may occur resulting in poor quality coffee with onion-like flavor or even "stinker" beans.[49] Microscopic observations of defective beans revealed bacteria (*Bacillus brevi*) in stinker beans, yeasts and molds (*Eurotium*) on beans with an earthy flavor, and molds (*Aspergillus*) and bacteria on beans with a moldy flavor.[22] The dried coffee beans are hygroscopic and mold growth with the formation of off-flavors may occur when the drying of "natural" coffee is not well controlled.[84]

"Washed" coffee is also sensitive to moisture and moisture levels over 12% to 13% during storage and distribution may lead to moldy beans.

The microbial load on raw and roasted coffee was analyzed by Mohr.[59] Raw coffee beans had microbial counts of 5–8 × 10^4 g^{-1}. Freshly roasted coffee was nearly sterile, whereas retail roasted coffee had counts up to 4–7 × 10^3 g^{-1} indicating recontamination after roasting. Coliforms, yeasts, and molds were not observed. The microflora of instant coffee consisted of over 90% of lactobacilli with *L. plantarum* and *L. brevis* as the predominant organisms.[90] The lactobacilli apparently develop in the wet concentrate before it is dried. *Lactobacillus plantarum* was able to grow in the presence of 50% total coffee solids but was inhibited by adding 15 mg pure caffeine ml^{-1} to the concentrate. Growth of *L. plantarum* in reconstituted coffee gave off-flavors and a reduction in pH from 5.2 to 4.4. *Escherichia coli, Salmonella* spp., *S. aureus,* and *B. cereus* were unable to grow in 2% solutions of decaffeinated and nondecaffeinated coffee and died within 24 hours.[90]

Pathogens

The presence of food poisoning bacteria has not been reported for coffee beans. Toxigenic molds such as *Aspergillus ochraceus, A. flavus,* and *A. versicolor* and *Penicillium cyclopium, P. citrinum,* and *P. expansum* are common on green coffee beans.[58] Mycotoxin formation in coffee beans has been studied extensively. Aflatoxin has not been found in the past[31] due to the presence of caffeine, which inhibits growth of *Aspergillus parasiticus* and the formation of aflatoxin.[13] The same is true for sterigmatocystin.[11] Nevertheless, trace amounts (ng kg^{-1} and not μg kg^{-1}) of aflatoxin B1 were recently found in Arabica coffee from Yemen (2.4 to 20.2 ng kg^{-1}) and robusta coffee from Indonesia (2.9 to 32.9 ng kg^{-1}).[61] Trace amounts of ochratoxin A (OA) are often detected. Studies on the fate of OA during roasting are inconclusive. An early US study concluded that the incidence of contamination of the green beans is low, that 70% to 80% of the toxin is destroyed during roasting, and therefore the probability of finding OA in a coffee drink is very low.[48] In an Italian study, 58% of green coffee samples were contaminated with OA at 0.2 to 15 μg kg^{-1} but roasting gave significant destruction (from 48% to 100%) and brewed coffee contained no residual toxin.[55] However, recent studies[79] on the effect of roasting on the destruction of OA (initial levels 1.2 to 56 μg kg^{-1}) gave reductions of only 30%, and OA was found in 16 out 40 samples of brewed Swiss coffee; amounts were low and only 4 samples had values over 2 μg kg^{-1} (maximum 7.8 μg kg^{-1}). A survey of 291 samples of green (unroasted) coffee imported into the United Kingdom (UK) showed that about 200 were not contaminated with OA and only low levels were detected in the rest. Only three samples had more than 10 μg kg^{-1} and levels were not considered to be of concern.[51] A proposed EU limit of 4 μg kg^{-1} is under discussion.

36.4.4 Preservation

Properly processed beans with a moisture content of 12% are microbiologically stable. However, a value of 12% moisture is close to the limit of acceptability, because depending on the coffee variety and temperature, the water activity is 0.60 to 0.77. At such relatively high water activities, small temperature changes during transportation may result in local condensation and mold growth.[17]

Studies with wet-processed Arabica coffee stored at 20°, 25°, 30°, and 35°C at 60%, 75%, 85%, 90%, and 95% relative humidity (RH) gave rapid mold growth at 85% RH and above and slow growth at 75% RH especially at higher temperatures.[60] This and other studies indicate that storage of green beans at 75% RH results in coffee with acceptable organoleptic quality even when there is some development of molds, because the latter are inactivated during roasting. A moisture content of 15% for green beans is considered a practical upper value above which quality problems may occur.

Coffee has antibacterial activity against a range of pathogens including *S. aureus, V. parahaemolyticus, V. cholerae,* and *Salmonella*.[52,78,87] The antibacterial effects have been attributed to tannins and caffeine but the main effects are due to the degree of roasting of the coffee beans and the chemical compounds resulting from the Maillard reaction, caramelization, and thermal decomposition. The inhibitory activity could be demonstrated in regularly prepared coffee extract.[19,20]

The antimicrobial activity of coffee does not prevent the flat-sour spoilage of sterilized canned liquid coffee by thermophilic anaerobes.[62,63] The organisms were identified as *Desulfotomaculum nigrificans* and *Clostridium thermoaceticum*. Closer identification suggests that the reported *D. nigrificans* isolates also were strains of *C. thermoaceticum*.[64] Spores of *C. thermoaceticum* were extremely heat-resistant with $D_{120°C}$ of 6 to 25 minutes and $D_{121°C}$ of 44 minutes.[54,63] Sugar has been identified as a source of these spores.[82] For canned liquid coffee, which is sold hot from vending machines, suitable raw materials and a sterilization process of F_0 15 to 20 are recommended.

36.5 CONCLUDING REMARKS

Tea and coffee have in common an excellent public health status because of the absence of microbiological hazards in the materials and the use of boiling water in their preparation. Herbal teas have the same good reputation, however, they typically have a high microbiological load, which may include organisms of public health significance. Preparation with boiling water is thus recommended to ensure the safety of the prepared drink. Longer term, the microbial contamination of herbal tea raw materials should be reduced by the application of good agricultural practice (GAP) in growing and harvesting of the raw materials. The European Herbal Infusions Association is implementing GAP through a Code, which is available in various languages, to support hygienic practices in the countries of origin of herbal teas.[28]

For tea there is growing evidence that it is not only a safe and refreshing drink but tea may also have beneficial health effects. For example black and green tea contain substances that have *in vitro* very strong antioxidant activity, and these flavonoids are expected to have anticarcinogenic properties and to reduce coronary heart disease. This is a topic that will be actively researched in the near future, in particular for green tea, which has the higher level of antioxidants.[83,86] A recent CRC special issue: *Tea and Health* provides an overview of the scientific information to date.[18]

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III

Foodborne Pathogens

37

Surveillance of Foodborne Disease

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37.1 INTRODUCTION

Surveillance has been defined "as the continuous, systematic collection, collation and analysis of data and the prompt dissemination of the resulting information to those who are empowered to take appropriate action,"[3] and is an essential element of any food safety program, providing data on the prevalence of foodborne infection in man, in food-producing animals, and throughout the food chain.

Foodborne infections have been a major cause of human disease for centuries, yet they remain greatly under-reported and their true incidence unknown. Various studies have indicated that foodborne diseases may be approximately 300–350 times more common than reported cases indicate. It is only within the past 20 to 25 years or so that there has been a renewed awareness of their public health importance and the need to develop surveillance programs. Some impetus was given during the 1980s for improved surveillance following major outbreaks of listeriosis in Switzerland,[15] the United States (US),[53,87] and Canada.[146] Surveillance of foodborne disease since has been increasingly developed in many countries worldwide with varying national resources determining the extent and sophistication of programs undertaken. The World Health Organization (WHO) has drawn attention to foodborne disease as a worldwide problem and the role of surveillance as an essential step toward the introduction of control measures.[188]

Foodborne disease also has acquired a further international dimension with outbreaks affecting tourists from northern hemisphere countries holidaying in the Caribbean, the Mediterranean littoral, Southeast Asia, and elsewhere. In Scandinavia, for example, routine surveillance has indicated that between 78% to 86% of salmonella cases and 60 to 70% of campylobacter cases reported annually involve tourists returning from such areas.[121] In addition to outbreaks affecting tourist groups, other outbreaks have been reported from northern Europe and North America associated with imported foods such as chocolate, bean sprouts, salami sticks, savory snacks, eggs, pasta, and cheese.

While recent decades have seen many advances in food technology and food hygiene standards and the introduction of numerous legislative control measures, the incidence of reported foodborne disease has increased significantly in most countries, particularly since the early 1980s. Statutory notifications of food poisoning in the United Kingdom (UK), for example, increased four-fold between 1982 and 1994, with the number of laboratory reports of salmonellosis, campylobacter enteritis, verotoxin-producing *Escherichia coli* (VTEC), and other foodborne infections increasing in parallel.[122] A 1989 review in North America drew attention to the increase in reported infection due to *Salmonella enteritidis*, *Campylobacter*, and VTEC, and to the increasing health care and litigation costs of foodborne disease.[169]

Greater awareness among primary care physicians, the availability of new laboratory technologies, improved reporting, and the development of national and international surveillance programs have undoubtedly contributed to these increases. Enhanced laboratory identification and isolation techniques have resulted in the reporting of "new" foodborne diseases such as campylobacter enteritis, viral gastrointestinal infections, and VTEC, while the increasing use of computerized databases has undoubtedly enhanced the comprehensiveness of surveillance programs. Other factors such as periodic media publicity in relation to salmonella and eggs, VTEC ("hamburger disease") and listeriosis, for example, may also have affected the number of samples submitted to laboratories by physicians, thus further influencing reported incidence rates. While much of this may be due to increased awareness, detection, and reporting, nevertheless a real increase in incidence, in particular of foodborne infections of animal origin (eg, salmonella, campylobacter, VTEC) would appear to have taken place in many countries.

Surveillance programs have identified problems associated with specific areas of food production (eg, dairy, shellfish, egg-laying) and catering (eg, airline, sandwich bars), and among specific population groups such as school and nursery children,[47] the elderly,[42] hospital patients,[26] farm workers,[54] and other consumers of raw milk.[150] Local surveillance in several urban areas in the UK in the early 1990s identified an epidemiological association during the early summer weeks between campylobacter illness and the consumption of milk from bottles delivered to doorsteps, the metallic caps of which had been attacked by wild birds, in particular jackdaws and magpies.[71]

The most important objectives in surveillance are:

1. the detection of increases in incidence or changes in the pattern of disease, thus enabling early investigation of outbreaks
2. the monitoring of trends to assess the need for intervention or to evaluate the effect of control measures.[57]

To measure the incidence, prevalence, and trends of any disease condition (acute or chronic, communicable or noncommunicable, foodborne or nonfoodborne), it is necessary to obtain and analyze relevant epidemiological data. The three basic steps in any surveillance program therefore consist of:

1. the regular reporting and collection of data
2. the collation and analysis of data
3. the dissemination of the information

The introduction of computerized databases and electronic communication systems in foodborne surveillance has greatly enhanced the collation, analysis, and distribution of epidemiological data, nationally and internationally.

37.2 REPORTING AND COLLECTION OF DATA

37.2.1 General Review

Surveillance may be undertaken at regional, national, or international level, with the collection of data obtained by passive (the most common) or active methods, depending on perceived importance and the degree of urgency in obtaining epidemiological information on specific disease problems. Considerable differences also may exist as to which foodborne infections (or disease syndromes) are regarded as of sufficient importance to be included in individual national reporting systems (Table 37–1).

While salmonellosis predominates as the most commonly identified infection in most countries, other foodborne diseases vary widely in their reported incidence. Outbreaks of food poisoning due to enterotoxin-producing *Staphylococcus aureus* and *Clostridium perfringens* continue to be reported commonly in most of continental Europe[121] (Table 37–2) and predominate in Latin American countries such as Cuba and Brazil.[172] Brucellosis is prevalent in most Mediterranean countries, while trichinellosis and botulism are public health problems in much of Eastern Europe. Campylobacteriosis, the most frequently reported cause of human gastroenteritis in the UK, the Netherlands, Canada, Australia, New Zealand, and in some regions of the US, remains uncommonly

Table 37–1 Foodborne Diseases Statutorily Notifiable to Health Agencies in Selected Countries (December 1997)

	ALB	AUS	CAN	CZE	FRA	GER	IRE	ISR	ITA	NL	NZ	POL	ROM	SPA	SWE	UK	US
Disease syndromes																	
Foodborne infections/ intoxications*																	
- bacterial	+			+						+				+			
- other	+							+	+	+				+			
Food poisoning*							+	+			+					+	
Gastroenteritis—under 2 yrs							+										
Hemolytic uremic syndrome		+															+
Infectious enteritis/ enterocolitis				+		+							+				
Intestinal infections of unknown origin	+			+													
Foodborne outbreaks		+			+					+	+			+	+		
Specific infections																	
Botulism			+	+	+	+			+	+	+	+	+	+	+		+
Brucellosis			+		+	+	+		+		+		+	+			+
Cholera	+	+	+	+	+			+		+		+	+		+	+	+
Campylobacteriosis		+	+	+		+		+		+	+				+		
Clostridium perfringens						+				+				+			
E. coli 0157/VTEC/EHEC			+								+				+		+
Listeriosis		+	+								+				+		
Salmonellosis	+	+	+	+	+	+	+	+	+		+	+	+	+	+		+
Shigellosis/bacillary dysentery	+	+	+	+		+	+	+	+	+	+	+	+		+	+	+
Staphylococcal enteritis						+				+		+		+			
Trichinellosis			+			+						+	+	+			+
Typhoid/paratyphoid	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Viral hepatitis (A)		+	+	+		+		+			+	+	+	+	+	+	+

*"Foodborne infections/intoxications" and "food poisoning" are usually synonymous, with the official terminology varying from country to country.

Key to country codes used:

ALB–Albania
AUS–Australia
CAN–Canada
CZE–Czech Republic
FRA–France
GER–Germany
IRE–Ireland
ISR–Israel
ITA–Italy
NL–Netherlands
NZ–New Zealand
POL–Poland
ROM–Romania
SPA–Spain
SWE–Sweden
UK–United Kingdom
US–United States

Source: Adapted with permission from *WHO Surveillance Programme for Control of Foodborne Infections & Intoxications in Europe*, Sixth Report 1990–1992, © 1995, Federal Institute for Health Protection of Consumers and Veterinary Medicine, and data from other national health agencies.

reported elsewhere. Infection due to *Campylobacter* usually occurs as sporadic cases, and outbreaks are relatively infrequent. VTEC infection (mainly due to *E. coli* serogroup O157), first recognized in Canada in the late 1970s, until recent years was reported infrequently by countries outside North America and the UK.

Most data collection systems are based on the laboratory reporting of isolates of foodborne pathogens; the reporting by physicians of prescribed infections (eg, botulism, cholera, listeriosis, typhoid) or clinically based disease syndromes (eg, food poisoning, infectious enterocolitis, gastroenteritis, hemolytic uremic syndrome); and/or the epidemiological investigation of outbreaks. Other data sources increasingly being used include physician "sentinel" and community studies (sections 37.2.4 and 37.2.5). Surveillance of foodborne disease can further be complemented via microbiological data obtained from (1) routine or ad hoc monitoring of foods (milk, water) by local health authorities from within the food industry (poultry broiler, dairy farming) or from surveys of specific at-risk foods (cold meats, shellfish, soft cheeses, pâté, salads), (2) monitoring of abattoir effluents, and/or (3) veterinary reports of sick food animals.

As a consequence of increased international awareness of the health and economic effects of foodborne disease, in 1980 WHO implemented the multinational Surveillance Programme for the Control of Foodborne Infections and Intoxications in Europe, the aims of which are

- identification of the causes and epidemiology of foodborne diseases in the European region of WHO
- distribution of the information gathered and collated to those concerned
- cooperation with national authorities in their efforts to strengthen the prevention and control of foodborne diseases (Table 37–2).

Table 37–2 WHO Foodborne Disease Surveillance Programme in Europe—Top 10 Etiological Agents Reported in Outbreaks by Participating Countries, 1990–1992

	Number of Outbreaks	(%)
Salmonella spp.	9,882	(84.5)
Staphylococcus aureus	409	(3.5)
Clostridium perfringens	356	(3.0)
Trichinella	181	(1.5)
Mushrooms	152	(1.3)
C. botulinum	123	(1.1)
Bacillus cereus	113	(1.0)
Campylobacter spp.	85	(0.7)
Shigella spp.	64	(0.5)
Escherichia coli	33	(0.3)
Others*	301	(2.6)

*others include viruses (60 outbreaks), chemical substances (54), scombrotoxin (48), *E. coli* 0157 (12)

Source: Reprinted with permission from *WHO Surveillance Programme for Control of Foodborne Infections & Intoxications in Europe*, Sixth Report 1990–1992, © 1995, Federal Institute for Health Protection of Consumers and Veterinary Medicine.

The WHO program has two component parts: annual reporting by participating countries and an adhoc early warning system relating to international foodborne disease emergencies, such as outbreaks associated with foods imported from one country to another. The Food and Agriculture Organization (FAO)/WHO Collaborating Centre of the Federal Institute of Health Protection of Consumers and Veterinary Medicine, Berlin, serves as the coordinating center, collating national and international data on foodborne disease. Despite the considerable political change in recent years in Eastern Europe, with disruption of national health systems, surveillance, and reporting of foodborne disease, by 1998, 38 countries were contributing to the program.

A similar international collaborative reporting system has been developed in Australasia, where the Communicable Disease Control Network of Australia and New Zealand, based on a semiformal agreement between the two countries, routinely exchanges epidemiological data.

Foodborne disease was defined for the purposes of the WHO program as "a disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water" and an outbreak as "an incident in which two or more persons experience a similar illness after ingestion of the same food, or after ingestion of water from the same source, and where epidemiological evidence implicates the food or water as the source of the illness" (Exhibit 37–1).

Despite agreement in principle to accept these definitions, they are not always fully complied with by individual countries within Europe. In some countries, outbreak reporting is required only when three or more people have been affected. Further differences also exist. In some countries a disease is regarded as being foodborne only when the causative agent has been identified in both affected individuals *and* in the food consumed. Some countries accept epidemiological evidence, and others include all reported cases of acute diarrheal disease regardless of whether it is foodborne or not. Comprehensiveness

Exhibit 37–1 WHO Definitions of Foodborne Disease

Foodborne disease—a disease of an infectious or toxic nature caused by or thought to be caused by the consumption of food (or water).

Single (sporadic) case—one case, as far as can be ascertained, unrelated to other cases in respect of consumption of food (or water).

Household outbreak—an outbreak affecting two or more persons in the same private household, not apparently connected with any other case or outbreak.

General/community outbreak—an incident in which two or more persons experience a similar illness after ingestion of the same food (or after ingestion of water from the same source), and where epidemiological evidence implicates the food (or water) as the source of illness.

Source: Reprinted with permission from *WHO Surveillance Programme for Control of Foodborne Infections & Intoxications in Europe*, Sixth Report 1990–1992, © 1995, Federal Institute for Health Protection of Consumers and Veterinary Medicine.

of investigation and reporting also varies considerably within and among different countries, largely due to differences in perceived priorities and commitment to surveillance. Comparability of collected data thus presents considerable problems.

It has been estimated that officially recorded data probably represent less than 10% (tip of the iceberg) of the real incidence of foodborne disease occurring in the community in countries with developed surveillance systems (Figure 37–1). Whereas the more clinically severe infections (eg, typhoid, listeriosis, botulism) usually require admission to hospital and thereby are more likely to be diagnosed, reported, and recorded, most others (eg, non-typhoid salmonella, campylobacter) may be self-limiting with a smaller proportion of cases seeking medical attention. In a study relating to problems affecting the diagnosis of foodborne disease in Wales, only 1 in 26 people suffering an acute episode of gastroenteritis consulted their family physician.[105] Furthermore, the physician may not arrange for a fecal specimen to be sent for laboratory examination or to notify the local health authority. Viral infections in particular are very much under-reported and the responsible etiological agent is even less likely to be identified. Not only is the illness usually of relatively short duration with the patient recovering within 24 to 48 hours, but even when a viral diagnosis is considered, it is essential that fecal specimens are sent to the laboratory within 24 to 48 hours of onset of symptoms. Viruses are cleared from the stool within 1 to 2 days and identification depends on the availability and utilization of electron microscopy or detection methods based on polymerase chain reaction (PCR) assays (see Chapter 52).

The disparity in the national salmonella isolation rates recorded in the UK (22 to 59 per 100,000 population) and in the US (13 to 22 per 100,000) between 1984 and 1995 appears to be due to differences in the comprehensiveness of reporting by laboratories to the respective national databases through the Public Health Laboratory Service (PHLS) Communicable Disease Surveillance Centre (CDSC), London; the Scottish Centre for Infection and Environmental Health (SCIEH), Glasgow; and the US Centers for Disease Control and Prevention (CDC), Atlanta, rather than to any major differences in disease incidence. An even greater disparity is seen for campylobacter infection with reported UK national isolation rates of 40 to 84 per 100,000[122] compared with reported US national rates of 5 to 6 per 100,000.[124,163] However, in the US, where laboratories in some areas of the country were routinely screening diarrheal feces for *Campylobacter*, isolation rates up to 71 per 100,000 were recorded.[16,124,149]

Within the UK during the 1970s and early 1980s, higher salmonella rates were observed in Scotland (33 to 51 per 100,000) than in England and Wales (21 to 31 per 100,000) and Northern Ireland (NI) (4 to 6 per 100,000). In Scotland, integrated interdisciplinary salmonella surveillance with isolates of all *Salmonella* species from medical, veterinary, and commercial (eg, poultry broiler industry) sources processed by one reference laboratory (the Scottish Salmonella Reference Laboratory, Glasgow) has been a feature since the early 1970s (Figure 37–2). Concurrently with the increasing awareness of the emergence of infection by *S. enteritidis* phage type 4 throughout the UK, the number of reports of enteropathogens received by CDSC from hospital laboratories (ie, outside the PHLS) in England and Wales doubled between 1985 and 1995. During this time, the different sources of information on human salmonellosis in England and Wales were amalgamated into a single data set.[55] Salmonella isolation rates comparable with those recorded in Scotland were reported thereafter.[122]

Surveillance programs for infection due to *E. coli* O157 have been increasingly developed in recent years in many countries, in some of which (eg, Australia, Canada, New Zealand, Sweden, US) the disease has been made notifiable. Surveillance of the hemolytic uremic syndrome (HUS) and non-O157

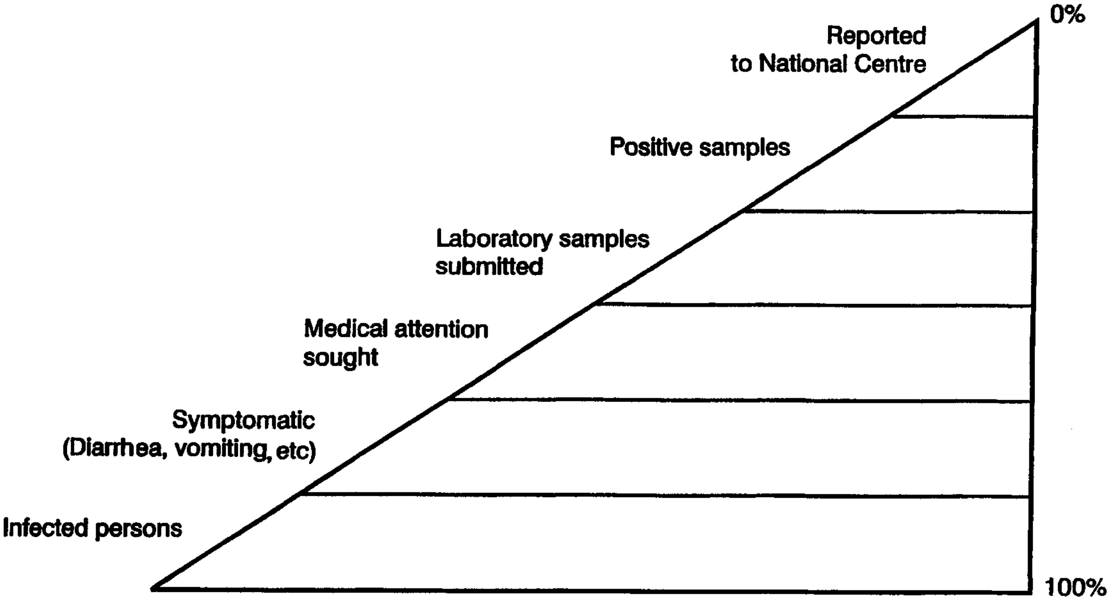

Figure 37–1 Foodborne Disease "Iceberg"—Estimated Prevalence in the Community

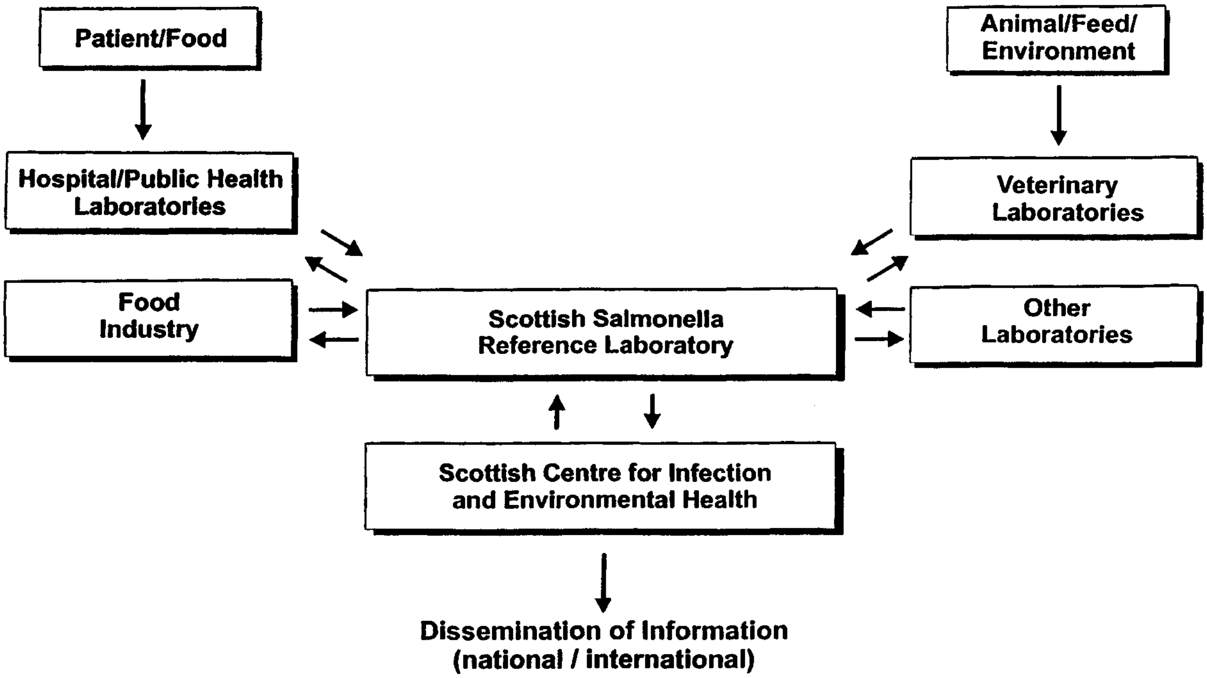

Figure 37–2 Salmonella Surveillance in Scotland—A Schematic Representation

VTEC infections due to serogroups that ferment sorbitol (and therefore are unlikely to be detected by the routine use of sorbitol-MacConkey [SMAC] medium), however, have been less well developed to date.

Epidemiological data sources used in surveillance can differ considerably from country to country. In the UK, the three main sources for reports of foodborne disease are: (1) notifications of food poisoning, (2) the reporting of laboratory-confirmed infections, and (3) the reporting of general outbreaks of infectious intestinal disease. In England and Wales, the data from all three sources are coordinated by CDSC[181] and in Scotland by SCIEH. Within Wales, a three-tier arrangement (local, regional, and national) for communicable disease surveillance, CoSurv, based on laboratory-confirmed infections, has been developed that links laboratories, public health, and environmental health departments with each other and with the regional and national surveillance centers based in Cardiff and London. CoSurv, has now been installed in all PHLS laboratories and regional surveillance centres throughout England and Wales.[66] In 1997 a collaborative project was initiated involving CDSC and SCIEH toward the development of an enhanced standard surveillance system for the investigation and reporting of cases of food poisoning throughout the UK.

In the US, the collection of surveillance data has been based primarily on state and federal legislative requirements to report prescribed foodborne diseases, in parallel with voluntary reporting to CDC of outbreaks as well as specific infections (eg, listeriosis, campylobacteriosis). Similar legislative reporting arrangements also are featured in Australia, Canada, and New Zealand. In January 1996, as part of a wider strategy in the US (Emerging Infections Program), a foodborne diseases active surveillance network (Food Net) was implemented by CDC in several states (Minnesota, Oregon, California, Connecticut, Georgia), the objectives of which are to: (1) determine more precisely the burden of foodborne diseases in the US, (2) determine the proportion of specific foodborne diseases associated with certain foodstuffs or with other exposures, and (3) provide the framework to respond rapidly and collaboratively to emerging foodborne diseases.[149] Surveillance was extended in January 1997 when laboratories in several counties in Connecticut and Georgia joined Food Net.

In the Netherlands, two official notification systems exist:

1. the mandatory reporting of notifiable diseases and outbreaks involving two or more people to the chief medical inspectorate
2. the reporting of complaints by individual members of the public, concerning suspected food contamination, to regional food inspection services.

In recent years, however, increasing reliance has been placed on physician sentinel reporting (Section 37.2.4). In France, surveillance is based on data obtained through the French Communicable Diseases Computer Network (Le réseau national téléinformatique de surveillance et d'information sur les maladies transmissables) initiated in 1984, involving approximately 50 sentinel laboratories and 500 primary care practices across the country.[25,177] Collaborating physicians, laboratories, and local and national health departments connected to the network each have their own computer terminals (provided free of charge to participating physicians) with the potential of exchanging information on a 24-hour basis. Electronic bulletins summarizing surveillance data and regional statistics are produced weekly.

Surveillance systems differ in their strengths and weaknesses (Table 37–3) and need to be complemented by other reporting mechanisms to obtain the most meaningful epidemiological data. Notification of foodborne disease, despite specific legislative requirements, is not always complied with. In westernized countries, most cases of typhoid or shigellosis are spread by person-to-person rather than by food- or waterborne transmission, while in some instances campylobacteriosis, VTEC, and salmonellosis are due to zoonotic contact with infected animals or with a contaminated environment. Syndrome reporting (food poisoning, gastroenteritis) usually is based on a clinical opinion of the likely nature of the illness, which in fact may sometimes not be of microbiological origin or due to foodborne spread. Laboratory-based systems have a greater cost implication and are subject to selection bias being dependent on appropriate clinical specimens (feces, blood) being submitted by physicians and others, being successfully processed and the results reported to relevant regional or national surveillance centers. Outbreak investigation data vary widely in the comprehensiveness and quality of the investigation undertaken and the information provided, much of which is dependent on the interest and enthusiasm of individual investigators. Hospital admission data relate only to more seriously ill cases requiring hospital treatment. Physician sentinel and community

Table 37–3 Foodborne Disease Surveillance Reporting Systems: Basic Strengths and Weaknesses

System	*Strengths*	*Weaknesses*
Statutory notification	Legislative requirement. Clinical/laboratory evidence. Opportunity of early alerting by telephone, e-mail. May include confirmatory laboratory evidence (see also below).	Variations in comprehensiveness of physician diagnostic accuracy, regularity, and timeliness of notifying. Often unsupported by confirmatory laboratory evidence.
Reporting by hospital, public health (or other) laboratories	Confirmation of infectious or toxic agent. Further identification of etiological agent (serotyping, toxin-testing). High reporting level where good local/national collaboration. Detection of outbreaks. Monitoring trends.	Dependent on appropriate clinical material being submitted by physicians, etc. Policy and cost limitations in the range of culture methods. Specificity of diagnostic test(s). Time lag (7 days +) between onset of illness and report.
Physician sentinel studies	Front-line picture of clinical events. Comprehensive of those people seeking medical attention within participating practices.	Dependent on affected person seeking medical attention. Selected practices only. May or may not have confirmatory laboratory evidence.
Community studies	Truer estimate than sentinel studies of real extent of disease incidence in the community. Complemented by laboratory testing for possible etiological agent.	Dependent on collaboration of those persons participating. Undertaken over a limited time. Selected communities, expensive and time consuming.
Hospital admissions	Clinical evidence. Can provide early warning of an acute foodborne disease incident.	More seriously ill cases only likely to be admitted to hospital. Dependent on epidemiological awareness by hospital medical staff and good professional relationships with public health colleagues.
Outbreak reporting	Identifies occurrence of circumscribed outbreaks and enumerates numbers affected. Provides information on suspected (or confirmed) food vehicle(s), etiological agents, and reasons for outbreak.	Comprehensiveness of investigation and quality of data obtained dependent on the interest and enthusiasm of the investigator.

studies are expensive and time-consuming to organize and administer and most usually are undertaken over a limited time period. Passive surveillance is less costly than active surveillance, but is also less complete.

37.2.2 Statutory Reporting (Notification)

Statutory reporting (notification) of foodborne disease(s) by microbiologists or physicians is a legislative requirement in most countries, the primary purpose of which is to alert the public health authorities (preferably by telephone or by electronic means) so that the investigation of outbreaks and other incidents and appropriate preventive action can be undertaken as soon as possible. Data obtained also are used for longer term surveillance purposes at regional, national, or international levels (Figure 37–3). The collected data may take the form of reports of isolates of specific foodborne pathogens (*Salmonella* species, *Campylobacter* species) by laboratories or as syndrome reporting (food poisoning, gastroenteritis, infectious enterocolitis, HUS) by physicians, but the situation can vary from country to country. Within the US, Canada, and Australia, for example, legislative requirements can differ also from state to state or province to province compared with those required under national legislation. The comprehensiveness of reporting also can differ widely within individual countries from region to region, from

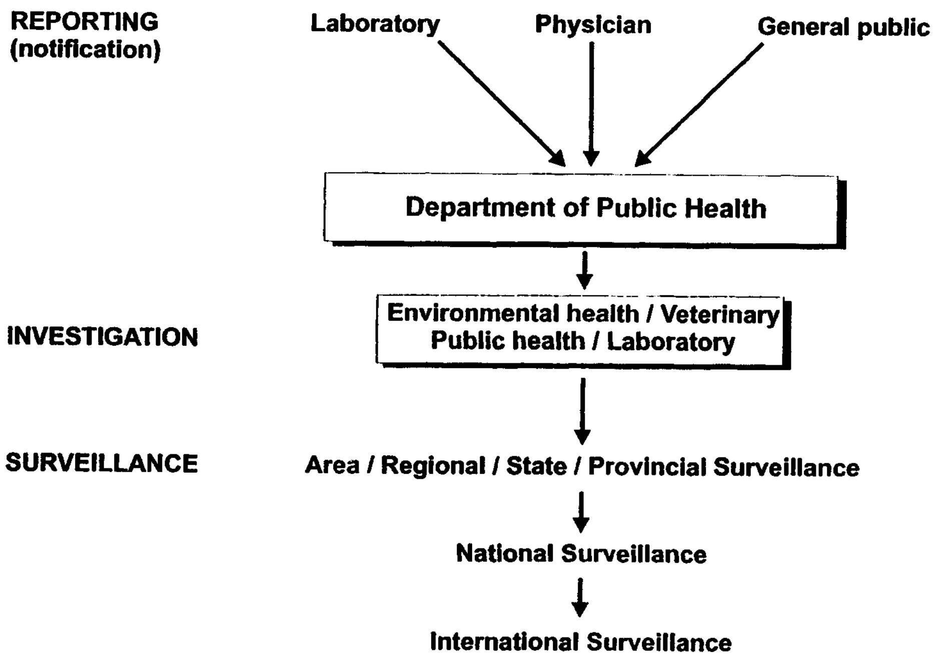
REPORTING (notification)
Laboratory
Physician
General public
Department of Public Health
INVESTIGATION
Environmental health / Veterinary Public health / Laboratory
SURVEILLANCE
Area / Regional / State / Provincial Surveillance
National Surveillance
International Surveillance

Figure 37–3 Reporting, Investigation, and Surveillance of Foodborne Disease

laboratory to laboratory, and from physician to physician. In some countries, for example, Cuba, the reporting of foodborne diseases is mandatory at all levels of the health system.[172]

In the UK, physicians have a statutory duty to notify the local health authority of cases (or suspected cases) of food poisoning, for which a fee (£2.30/$3.86 in 1998) is payable. Cholera, typhoid, paratyphoid, and viral hepatitis are also similarly notifiable, whether or not thought to be foodborne. Laboratory confirmation of an etiological agent is not necessarily required, although it may be available in many instances. In the absence of formal notification by a physician, food poisoning data can also include cases ascertained by other means such as in the course of an outbreak investigation or as a result of complaints by members of the public.

In North America, notification of specific foodborne diseases is mandatory within individual states and provinces. In the US, onward reporting of notifiable diseases by state health departments to the CDC is undertaken on a voluntary basis through the computer-based National Electronic Telecommunications System for Surveillance (NETSS). In Canada, there are similar legislative requirements for the notification of foodborne diseases, initially to the provincial health department and thereafter to the Laboratory Centre for Disease Control (LCDC) in Ottawa. Although *E. coli* O157 infection and HUS have been notifiable in some provinces (eg, Alberta, British Columbia) since the early 1980s, it was not until 1989 that VTEC infection (hemorrhagic colitis) was added to the list of diseases under national surveillance. In the US, *E. coli* O157 came under formal surveillance nationally in 1993, in addition to which an increasing number of states (eg, New Mexico, New York, Ohio, Texas) have also included HUS as a notifiable disease.

In Australia, most states generally have consistent legislation relating to which foodborne diseases require notification. Only New South Wales does not require the reporting of campylobacteriosis, shigellosis, or yersiniosis. HUS in children under 16 years is reportable by microbiologists and nephrologists to the Australian Paediatric Surveillance Unit, as well as to state health departments in the Northern Territory, Tasmania, and Western Australia. Syndrome reporting such as food poisoning, gastroenteritis in an institution, or in a foodhandler, is also required in some states.

In New Zealand since July 1996, salmonellosis, campylobacteriosis, listeriosis, viral hepatitis A, and outbreaks of food poisoning are reported by physicians to local environmental health departments, although in many instances in urban areas, epidemiological investigations are still undertaken by public health board staff. Onward reporting of data is undertaken via a computerized network to the Institute of Environmental Science and Research Communicable Disease Centre at Porirua, and then by e-mail to Canberra, Australia, as part of the Communicable Disease Control Network of Australia and New Zealand. National guidelines on surveillance, reporting, and investigation of food- and waterborne diseases were produced by the New Zealand Ministry of Health in 1994, including specific guidelines for the surveillance of mollusk-associated (shellfish biotoxin) disease.

In the Netherlands, there are two categories of statutorily notifiable diseases: group A (typhoid fever), which is required to be reported on clinical suspicion and group B (botulism, cholera, bacillary dysentery, paratyphoid, and incidents of foodborne infection or intoxication), which require a confirmatory laboratory diagnosis. In addition, foodborne disease has to be reported if the diagnosis is suspected among food workers, among people professionally nursing or caring for other people, or if two or more persons have developed similar symptoms within 24 hours after consuming the same food (or drink). Information obtained from these official systems has, however, generally been of poor quality and usually only related to outbreaks; as a result, increasing reliance has been placed on physician sentinel reporting to provide more accurate data. Salmonellosis, a common cause of foodborne disease in the Netherlands, was removed from the list of statutorily notifiable diseases in 1985; since then data have been collated from laboratory, sentinel research, and community studies.

In most European countries other than France, surveillance is based primarily on statutory reporting, although the legislative requirements for individual diseases may differ considerably from country to country (Table 37–1).

37.2.3 Laboratory Reporting

Laboratory-based surveillance systems provide definitive data relating to the isolation of pathogens from the feces (or other clinical material) of affected humans, as well as from food animals, food, and the environment. Laboratory surveillance is particularly useful in detecting outbreaks and monitoring trends. The reporting of laboratory-confirmed foodborne infections, a legislative requirement in many countries, forms an integral part of most surveillance programs. The surveillance of specific infections (salmonellosis, listeriosis, VTEC, *C. perfringens*) is further complemented by, or in some countries fully based on, epidemiological data provided by specialist reference laboratories. Some laboratories, in particular commercially-based private laboratories, may vary considerably in the comprehensiveness of reporting to surveillance centres and/or in forwarding isolates to appropriate reference laboratories for further characterization.

In the UK, voluntary reporting by laboratories (national health service, university, and PHLS) has formed the basis of foodborne disease surveillance since World War II, in parallel with statutory reporting (notification) of food poisoning, typhoid, paratyphoid, and cholera. In the US, reporting requirements for laboratories differ by state. Reports of isolates of foodborne pathogens (*Salmonella* spp., *Shigella* spp., and *Campylobacter* spp.) are forwarded thereafter on a voluntary basis to CDC through a computer-based surveillance system via the Public Health Laboratory Information Service (PHLIS), independently of NETSS. Whereas NETSS records disease categories such as salmonellosis, the PHLIS system provides more specific data relating to serotype (*S. enteritidis, S. typhimurium*). In Canada, voluntary laboratory reporting of non-notifiable enteric pathogens (eg, enteropathogenic and enterotoxigenic *E. coli, Bacillus cereus, Yersinia, Aeromonas*) to LCDC also is done. In Australia, laboratory reports of individual enteropathogens (*Salmonella* spp. and others) are collected by the National Salmonella Surveillance Scheme in Melbourne, and those from virus laboratories are collected by the Commonwealth Department of Human Sciences and Health in Canberra.

On numerous occasions, laboratory-based surveillance has detected and led to the control of community outbreaks of foodborne disease. Among these were the *E. coli* O157 apple juice[6,14] outbreaks in western US and Canada; listeriosis in Switzerland,[15] France,[60] US,[53,87] Canada,[146] and the UK;[93] cholera in Maryland, United States;[165] paratyphoid fever in France;[40] Norwalk-agent gastroenteritis in Louisiana and other states (Maryland, North Carolina, Mississippi, Pennsylvania, Texas) in the US;[46,80] shigellosis in Scandinavia[77] and in the UK;[5] and national and international outbreaks of salmonellosis in North America[19,34,65,141] and the UK.[31,59,78,138]

Laboratory-based surveillance programs are dependent, however, on the appropriate pathological specimens being submitted for testing and being successfully processed in the laboratory, and the results forwarded to the surveillance center. A 1993 study in England showed that only 5.4% of adults who had experienced a recent episode of diarrhea reported that a fecal specimen had been requested for microbiological examination.[49] A disproportionate number of fecal (and other clinical) samples are submitted from young children compared with from older children and adults. Age-specific isolation rates therefore may readily become distorted unless computerized databases recording the number of specimens examined in relation to the frequency of isolates by patient age-group (denominator data) are used by laboratories.

37.2.4 Physician Sentinel Studies

Primary-care physician-based sentinel studies for gastrointestinal disease are relatively uncommon compared with those for respiratory infections, but are being developed increasingly for surveillance purposes in some countries, in particular in the Netherlands, France, Australia, and the UK.

In the Netherlands, the first sentinel study, involving 40 general practices, two regional public health services, and two regional laboratories, was undertaken in the late 1980s. All people with acute gastroenteritis seeking medical attention were asked to complete a short questionnaire and submit a fecal sample for laboratory screening for *Salmonella, Shigella,* and *Campylobacter.* An incidence rate of 15 episodes of acute gastroenteritis per 1,000 persons per year was recorded.[100]

In France, the Communicable Diseases Computer Network collects data on seven selected non-notifiable disease conditions from 500 primary care medical practices throughout the country. Acute diarrheal disease was added to the list in 1990. In 1992, the second full year of diarrheal disease surveillance, 103,800 cases were reported, equivalent to an annual estimated incidence of 5,500 cases of acute diarrheal disease per 100,000 of the French population.[25] Since 1995 the collected data have been available electronically on the worldwide network (Sentiweb).[17] Although not geared primarily to outbreak recognition, sentinel surveillance led to the detection of an extended outbreak of acute gastroenteritis across four regions of the country (Ile-de-France, Bretagne, Rhône-Alpes, Pays-de-Loire) over a 10-week period during 1994–1995.[52]

In England and Wales, the Royal College of General Practitioners (RCGP) developed a sentinel practice scheme during the 1980s involving 60 general medical practitioners (GPs) and about 425,000 patients, for a range of clinical conditions including infectious gastrointestinal disease. The practices report weekly to the RCGP, with the collated data returned weekly to participating practices. Since 1986 a network of volunteer general practices (GP Surveillance of Infection in Wales Scheme) has operated in Wales,[104] reporting weekly on nine clinically diagnosed infectious diseases, including acute infectious gastroenteritis since 1990 and diarrhea with frank blood since 1995.

Following UK government recommendations,[118] a more comprehensive, in-depth epidemiological study of Infectious Intestinal Diseases (IID) (irrespective of whether or not foodborne in origin) based on 70 general medical practices was undertaken in England between July 1993 and January 1996, involving the Medical Research Council Epidemiology and Medical Care Unit, the Epidemiology Unit at the London School of Hygiene and Tropical Medicine, and the PHLS. The objectives of the study were to:

- estimate the etiology and incidence of IID both in the population and presenting to GPs
- compare these numbers and etiologies with those recorded by the national surveillance system
- estimate the prevalence of asymptomatic infection with agents associated with IID
- document differences between cases of IID and similar but well people (controls)
- estimate the socioeconomic costs of IID.

Although the purpose of the study was to provide insights into the causes of IID, its design was not intended to monitor trends, but rather to provide a more accurate estimate of the relationship between the real incidence of IID and that detected by routine national surveillance.

In addition to the main study, there were four secondary studies to ascertain the number of IID cases presenting to hospital accident and emergency departments, the long-term medical sequelae of IID, the completeness of reporting of enteropathogens by laboratories in England to the national surveillance centre (CDSC), and the completeness of the ascertainment of IID by GPs. An interim report was published in 1996, followed by the final report in 1999.

A prevalence study in two urban general practices in Scotland indicated that 12% of people seeking medical attention had reported diarrheal symptoms involving some loss of time from school or work in the 3-month period prior to being interviewed.[157] A more recent study of acute gastrointestinal infection presenting to GPs across Scotland began in May

1996, embracing a population of about 150,000, with fecal samples examined for common enteropathogens by a single laboratory.

In Australia, the Australian Sentinel Practice Research Network, comprising 99 general practices throughout the country, reports weekly on 12 acute medical conditions including gastroenteritis. Although used in some Canadian provinces, sentinel physician reporting has not yet been developed to any extent in the US.[163]

37.2.5 Community Studies

Compared with physician sentinel studies, which are based on those people who seek medical assistance, more accurate assessments of the incidence of gastroenteritis can be provided by community studies, the first of which were undertaken in the US during the 1950s.

Community studies have been developed further in some European countries. A 1991 study in the Netherlands in four different geographic regions of the country involving a sample of about 1,500 persons randomly selected from each of the population registers of the four regions showed that of those who reported having experienced symptoms, only 20% had consulted a physician in person or by telephone.[69] A similarly structured community study was undertaken in England during 1993–1996 as part of the extensive IID epidemiological study.[173]

Although adding further to costs and administrative input, more definitive epidemiological data are obtained from studies supported by microbiological investigations.

37.2.6 Hospital Admission Records

Admission records of ill persons requiring treatment in hospitals, irrespective of whether or not a definitive clinical or microbiological diagnosis has yet been made, can provide useful early indication of the occurrence of acute gastrointestinal disease in the community. The first indication of an outbreak of foodborne disease[13,21–23,101,176] may be the admission to a hospital of groups of people presenting with similar clinical (diarrhea, vomiting, fever, HUS) or epidemiological histories (attended the same playgroup, members of the same social or ethnic group).

Hospital discharge records or death registration of individual patients also may provide useful, albeit limited, retrospective data of the more serious foodborne diseases (eg, botulism, VTEC).

37.2.7 Outbreak Reporting

Outbreak reporting is a fundamental feature of surveillance in most countries and in some (eg, the Netherlands, New Zealand, France, Spain) is a legislative requirement. Outbreak reporting data are, however, heavily dependent on the quality of the original investigations, the standardization of laboratory methods, and the consistency of reporting.

Although contributing within Europe since 1980, the surveillance of outbreaks in the UK has become standardized only since the early 1990s. In the US, CDC initiated national surveillance of foodborne disease outbreaks in 1966. Outbreaks most usually are reported to CDC by state and local health departments, although they may also be reported by federal agencies such as the US Food and Drug Administration (FDA), the US Department of Agriculture, and the US Armed Forces, or occasionally by private physicians; outbreaks occurring on cruise ships or associated with food eaten outside the US are not included. Summaries of reported outbreaks are published every 5 years.[12] The Canadian Foodborne Disease Reporting Centre of the Federal Health Protection Board, Ottawa, began collecting and publishing information on outbreaks in 1973.[171] These data are generally more complete than in the US because each of the 10 Canadian provinces and territories reports annually to Ottawa.

The WHO Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe, primarily based on outbreak reporting, has provided useful epidemiological data since 1980[116,121] despite varying levels of input by participating countries, differing national data collection systems and definitions of outbreaks.

37.2.8 Other Sources of Information

Foreign Travel

Although the data usually are heavily biased toward those infections that are laboratory-confirmed, information from returning travelers has been used effectively for surveillance purposes. Additional information can be obtained also through questionnaires issued to travelers and collected in flight, at airports, or in the primary care setting. Such studies can include targeted groups, for example people who visit travel clinics for advice before travel, and can help monitor the effectiveness of the information given.

Gastroenteric infections, most of which are food- or waterborne in origin, constitute the bulk of disease reported. A survey in Scotland showed that *Salmonella* spp. are the most common enteropathogen isolated from returning tourists and other travelers from overseas; other infections reported included typhoid, paratyphoid, shigellosis, campylobacteriosis, giardiasis, entamoebiasis, and hepatitis A.[179]

Medical services specifically for overseas travelers are developing in an increasing number of countries. Many organizations audit their work and publish data that are coordinated by the International Society of Travel Medicine, whose journal also highlights specific food-related risks such as the consumption of unpasteurized goat's milk cheese.[67]

The Food Chain

Routine and ad hoc monitoring of the food chain from the farm to the consumer by environmental health, veterinary, and food protection authorities (Exhibit 37–2) provides important data relating to food contamination. Microbiological monitoring of foods is based on samples (eg, milk, water) submitted by environmental health officers (EHOs), veterinary, or other trained staff. The professional group responsible for sampling differs from country to country. In the UK, EHOs are mainly responsible; veterinarians are responsible in most of the rest of Europe.

In England and Wales, the PHLS Food Surveillance Group coordinates regular surveys carried out by its food chain (sentinel) laboratories of at-risk foods such as poultry meat, sausage meats, sliced meats, shellfish, prepacked sandwiches and salads, soft cheeses, airline meals, and cook-chill foods.[58,99,108,132] Inspections of retail chicken have demonstrated that salmonella contamination rates of frozen birds sampled decreased from 79% in 1979 to 41% in 1994 and of chilled birds from 54% in 1987 to 33% in 1994.[123,134] A study of frozen chicken carcasses in a hospital kitchen in Scotland identified salmonella contamination in 45% of birds and 100% of the weekly consignments examined.[114] Surveys in other countries have demonstrated similar contamination rates.[76]

More recently, laboratory food-chain surveys in England and Wales by the PHLS and the Ministry of Agriculture and Food have been commissioned to assess the extent of microbiological contamination (*Salmonella* spp., *Campylobacter* spp., *Listeria* spp.) within catering and retail premises, in particular studying food items such as (1) salads and hors d'oeuvres from self-serve salad bars, (2) UK-produced raw chicken, (3) cream cakes, (4) raw cow's milk and cheeses, (5) ready-to-eat meats and meat products, (6) warm water shrimp, and (7) cooked, dried, fermented, and mechanically recovered meats.

Exhibit 37–2 Some Environmental and Veterinary Food Surveillance Mechanisms

- Local health authority monitoring of foods (milk, water, etc.)
- Commercial monitoring of foods (poultry industry, dairy industry, etc.)
- Laboratory surveys of foods (cold meats, shellfish, etc.)
- Veterinary investigation of "ill" food animals
- Sewage (human)/abattoir (animal) effluent surveys

Campylobacter contamination rates of up to 100% of birds examined, particularly fresh birds, have been demonstrated in surveys of retail poultry carcasses in several countries including the Netherlands[178] and the UK.[68] In Australia, regulations were introduced in Victoria in 1990 requiring the notification of isolates of *C. jejuni*, *L. monocytogenes*, or *Salmonella* species from food for human consumption. In New Zealand, following the 1993 biotoxin outbreak, a national shellfish surveillance program, administered jointly by the Ministries of Health and Agriculture and the New Zealand shellfish industry, has been applied to both commercial and domestic gathering.

With the decrease of border inspections within the European Union (EU), food inspection authorities are confronted with a greater challenge in the prevention of multinational foodborne outbreaks. To help combat such dangers, the European Community Coordinated Food Control Programme has the responsibility of supervising microbiological (and chemical) sampling and analysis of food involving all 15 countries in the EU.[27] It is also required of member states that food control authorities responsible for sampling and analysis "shall have (or have access to) a sufficient number of suitably qualified and experienced staff, in particular in areas such as chemistry, food chemistry, veterinary medicine, medicine, food microbiology, food hygiene, food technology and law." Within individual countries, input to the program is coordinated by national food coordinating committees. Results are collected nationally, with the data being forwarded to Brussels for collation. At the time of writing, results from this program have not been published. Surveillance based primarily on locally perceived problems within individual member states is also carried out as required by the relevant environmental health, veterinary, or other food protection agencies. The European Commission coordinates a Rapid Exchange of Information for Foodstuffs (RAPEX), which ensures that where a food hazard (microbiological or chemical) goes beyond individual EU national borders, all other member states are alerted so that they may take any necessary action at a national level. All laboratories in the EU engaged in official food control work are required to be accredited for that purpose by November 1, 1998.

Veterinary Surveillance

Statutory surveillance of salmonellosis and brucellosis in food animals is required in the UK under the Zoonoses Orders of 1975 and 1989 and requires the reporting of any isolate of *Salmonella*[125] or *Brucella* in any "food animal or bird or animal surroundings or feedstuff."

The recent emergence of transmissible spongiform encephalopathies as potential foodborne zoonoses has led to the introduction of legislation that makes them reportable not only in the UK but also in the rest of the EU.[28] Surveillance of the major foodborne zoonoses in both animals and humans also takes place at the international level. In Europe the appropriate authorities (whether medical or veterinary) are required to make an annual submission to the European Commission on trends, sources, and control of zoonotic infections.[29]

Surveys have demonstrated fecal carriage of human enteropathogens such as *Salmonella* species and VTEC by domestic farm animals, particularly cattle. Abattoir surveys have shown correlations between asymptomatic bovine infection and fecal contamination of carcasses. Veterinary studies in the UK[159,160,174] and in Canada[115] have also demonstrated a direct link between cattle and human *E. coli* O157 infection. Surveys of abattoir and sewage effluents have also been used to monitor the prevalence of *Salmonella* in food animals, in local human populations, and in the environment, providing an insight into circulating serotypes and phage types.[75,86]

Other veterinary and commercial surveillance sources, not always accessible by the health authorities, can also provide useful epidemiological data. Monitoring of poultry flocks, processing plants, and products within the poultry industry, for example, can provide a timely insight into the organisms present within a major food product and to which the human population may become exposed. Statutory monitoring in the UK as required by the Processed Animal Protein Order, 1989, identifies *Salmonella* that may be present in animal (and poultry) feed and subsequently in animal (and poultry) populations.

37.3 COLLATION AND ANALYSIS OF DATA

Following the receipt of reports from microbiologists, primary care and public health physicians, and veterinarians by national, state, or provincial surveillance centers as appropriate, epidemiological data are entered into a database for collation and analysis.

In the UK, data relating to statutorily notified cases of food poisoning in England and Wales are collected and collated by the Office of National Statistics (ONS) in London, in Scotland by the Information and Statistics Division (ISD) of the Common Services Agency of the Scottish Health Service, Edinburgh, and in Northern Ireland (NI) by the Department of Health and Social Security. In addition, the Infectious Diseases Surveillance Systems (IDSS), introduced initially in Wales in 1989 and subsequently in Scotland (SIDSS), are examples of computerized systems collating statutory notification (and other communicable disease) data, enabling rapid analysis of complex data sets, which, in conjunction with Epinet, have made possible the establishment of detailed, up-to-date national databases from health authority computerized data files. The development, for example, of CoSurv in Wales and the French Communicable Diseases Computer Network, further emphasizes the increasing importance of electronically operated databases in collation, analysis, and rapidity of feedback of data, an essential element of surveillance.

Automated statistical scanning of incoming laboratory reports has been developed by the PHLS in England and Wales which, by supplementing routine national surveillance, can facilitate the detection of outbreaks of foodborne disease at an earlier stage than is normally possible.[48]

The EU Salm-Net surveillance system, coordinated at the PHLS Central Public Health Laboratories, London, became operational in early 1994, with the primary aim of strengthening international laboratory-based surveillance relating primarily to incidents of human salmonellosis within the EU by creating an integrated on-line database available to all participating countries. The program, funded by the European Commission, comprises a network of microbiologists and epidemiologists responsible for national surveillance of salmonellosis, thereby facilitating the rapid exchange of information relating to outbreaks or the emergence of specific serotypes or phage types among participating countries. Salm-Net also aims to harmonize across Europe phage-typing schemes for *Salmonella typhimurium*, *S. enteritidis*, *S. virchow*, and *S. dublin*.[51] Since becoming operational, Salm-Net has facilitated the recognition and investigation of multinational outbreaks due to *Salmonella anatum*,[9] *S. agona*,[78] and *S. tosamanga*[64] respectively, as well as outbreaks of *Shigella sonnei*[5] and *E. coli* O157,[7] affecting several European countries. Funding has since been continued under the name of Enter-Net for an additional three years with an expansion of the remit to look at antibiotic resistance in salmonellae and to include VTEC in the surveillance system. Initiatives are under way to extend Enter-Net to become a more global network with the participation of countries in Eastern Europe, Africa, North America, and the Pacific Rim.

In the US, in addition to the development of Food Net, Pulse Net enables public health laboratories using Internet technologies to network with CDC as well as the Department of Agriculture and FDA. It allows public health scientists around the country to compare rapidly the DNA from bacteria involved in outbreaks of foodborne illness, in particular *E. coli* O157:H7.

Global salmonella surveillance is undertaken by the WHO Collaborating Centre based at the Central PHLS Laboratories, London, which collates and analyzes serotype frequencies reported by contributing countries.

37.4 DISSEMINATION OF INFORMATION

One of the essential components of any meaningful surveillance program is the feedback of analyzed data to contributing colleagues (clinicians, microbiologists, public health physicians, EHOs, veterinarians), administrators, and other interested people. This may be in the form of early feedback by telephone, fax, or electronically to alert health authorities as soon as possible of an emerging public health problem, the production and distribution of regular epidemiological bulletins or newsletters, or via published scientific papers.

The production of national (or regional) epidemiological bulletins has now become an important feature in most countries with established surveillance programs, whereby data and other relevant information are collated and published weekly, monthly, or quarterly for distribution nationally and internationally. In the UK, separate reports are produced within England and Wales (*Communicable Disease Report*), Scotland (*Scottish Centre for Infection and Environmental Health Weekly Report*), and Northern Ireland (*Communicable Diseases Monthly Report Northern Ireland*). Similar reports are also published (many in English) in the US (*Morbidity & Mortality Weekly Report*), Canada (*Canada Diseases Weekly Report*), Trinidad & Tobago (*Carec Surveillance Report*), Australia (*Communicable Disease Intelligence*), New Zealand (*New Zealand Public Health Report)*, France (*Bulletin Epidemiologique Hebdomadaire*), Denmark (*Epi-News*), the Netherlands (*Infektieziekten Bulletin*), Germany (*Epidemiologisches Bulletin*), and Spain (*Boletin Epidemiologico Seminal*) at weekly, fortnightly, or monthly intervals. Extracts from national reports are collated by WHO and published (in English and French) in the *Weekly Epidemiological Record*.

The WHO Surveillance Programme for the Control of Foodborne Infections and Intoxications in Europe to date (January 1999) has produced six reports, the first of which was published in 1981, and the most recent in 1995 covering the years 1990–1992,[121] collating and summarizing the data received from each of the participating countries in the European zone. The seventh report is currently being prepared. Periodic newsletters are also produced at more frequent intervals for distribution within collaborating countries.

National reviews summarizing the occurrence of outbreaks or the frequency of isolates of foodborne pathogens are produced (quarterly, annually, periodically) by various countries worldwide, particularly in Europe, North America, and Australasia. Reviews and analyses have also been produced within individual countries relating to specific enteropathogens such as *Salmonella*,[126,128] *Campylobacter*,[119,124,163] *Escherichia coli* O157,[61,154,158] and *Listeria monocytogenes*;[2,92] disease associated with areas of food production including the fish and shellfish[175] and dairy industries;[43,150,151] and hospital,[26,117] cruise ship,[82] airline,[132] and domestic forms of catering.[140] Publications relating to foodborne disease outbreaks and changing trends[41,92,100,127,171] and surveillance programs[121,153] have been produced also.

International publications *EVC News* and *Eurosurveillance* were introduced in Europe in 1996. Of these, the former is specific to VTEC infection and is produced (in English) by the Istituto Superiore di Sanita, Italy, as part of the European VTEC Club

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(EVC), collecting information on outbreaks, case-clusters, trends, and isolates of unusual strains. *Eurosurveillance*, a monthly publication, is produced (in English and French) by Hôpital National de Saint-Maurice, France, in collaboration with PHLS CDSC, UK, collating data from surveillance networks, reports of outbreak investigations, and other material of international interest within the EU. *Eurosurveillance* is one of several initiatives implementing EU policy, funded by the Directorate General of the EC, to increase the exchange of epidemiological information including the development of surveillance networks along with a training program for field epidemiology in Europe. A further development was the introduction in 1997 of *Eurosurveillance Weekly*, an electronic bulletin (in English) accessible on the World Wide Web or by e-mail, providing brief, authoritative, and up-to-date information on communicable diseases of European significance for public health personnel throughout the EU.

Within individual countries, supplementary epidemiological feedback has been developed by many health authorities (area, regional, provincial) in the form of newsletters circulated to local clinicians, microbiologists, infection control nurses, and EHOs, summarizing outbreaks and other items relating to foodborne (and other) infection of local, national, and international interest.

From October 1997, the EU has been piloting a European electronic network on communicable diseases through the Health Surveillance System for Communicable Disease (HSSCD), a component of the Interchange of Data between Administrations (IDA) program, building on the experience gained in the national surveillance undertaken in individual member states. Following evaluation in a number of countries (eg, Austria, England and Wales, Finland) and when the design is finalized, the network will be extended to other EU countries.[183]

37.5 THE USE OF SURVEILLANCE DATA

37.5.1 The Purpose of Surveillance

The primary role of surveillance is the detection of incidents of disease. Data obtained from surveillance programs, equally importantly, contribute to the longer term measurement of trends and the introduction of intervention or control measures (legislation, health education, media announcements, changes in food production, and processing methods) in the prevention of foodborne (and other) disease.

Surveillance → Information → Intervention → Prevention

Among the many examples of successful intervention following microbiological and epidemiological surveillance were the recognition of outbreaks of infection (salmonellosis, campylobacteriosis) associated with the consumption of raw milk, as a public health problem in Scotland throughout the 1970s and early 1980s.[150] Thereafter, control measures were implemented in 1983 in the form of legislation requiring the heat-treatment of all cow's milk for retail sale to the public[151] and in 1986 requiring that only pasteurized milk be provided to agricultural workers, and not unpasteurized milk as previously, as part of a wage-benefit.[54] Similar national legislation relating to the compulsory heat-treatment of cow's and goat's milk was introduced in Canada in 1991 following outbreaks of salmonellosis and other milkborne infections identified through provincial and federal surveillance programs. Outbreaks associated with the consumption of unpasteurized dairy produce in the UK (including cow's milk in some rural areas of England and Wales), the US, and other countries continue to be reported.[15,24,40,43,60,91]

Outbreaks of milkborne disease have also been reported due to failure to pasteurize properly or to postpasteurization contamination of milk and other dairy products (cheese, powdered milk),[9,10,35,95,111,138,152,154,176] the most extensive of which occurred in Illinois and adjacent states in 1985 affecting more than 16,000 people.[139] The importance of ongoing surveillance in monitoring control measures such as pasteurization and post-pasteurization hygiene at national and processing dairy levels should, therefore, not be minimized.

Surveillance programs also contribute to the recognition of specific epidemiological features of foodborne infection. Considerable differences exist in the incidence of reported disease in relation to the age and geographical distribution of those people affected, in seasonal incidence, and in the types of catering premises associated with outbreaks.

Age Incidence

Foodborne infections affect all ages more or less equally. Nevertheless, the highest reported incidence occurs among young children between 1 to 4 years of age. This age group, together with the elderly, are more likely to (1) have been more seriously ill, (2) have sought medical attention, and (3) have had a fecal (or other) sample submitted for laboratory examination. Infants under 1 year on conventional baby-feeding programs (breast or bottle) generally are less commonly exposed to foodborne disease, although community outbreaks associated with salmonella-contaminated powdered milks have been reported.

Seasonal Incidence

In temperate climates, the incidence of infections due to *Salmonella* and *E. coli* O157 increases noticeably during the summer and early autumn months. Campylobacter infections characteristically peak during late spring and early summer, some 6 to 8 weeks before the "salmonella season," followed in most years by a secondary autumn rise, for which there has been no satisfactory explanation, although ecological and agricultural factors may contribute. Foodborne viral (SRSV, Norwalk-agent) infections, particularly when associated with molluscan shellfish (oysters) farmed in sewage-polluted waters, are reported more frequently over the colder winter months, during which time the depuration (cleansing) process takes longer to be fully effective.

Catering Premises Associated with Outbreaks

Of 1,380 outbreaks of foodborne disease reported in Scotland between 1980 and 1985, 1,012 (73%) occurred as family outbreaks and 368 (27%) as general community outbreaks. The premises where food had been mishandled or contaminated were recorded in 500 outbreaks (36%); of these, commercial catering (hotels, restaurants, cafeterias) was implicated in 180 outbreaks (36%), domestic kitchens in 175 (35%), farms in 43 (9%), and hospitals in 27 (5%). The remaining outbreaks (15%) were associated with various other forms of catering (transport, old persons' homes, oil rigs).[153]

In Europe between 1990 and 1992, the place where food was contaminated or mishandled was reported by relatively few countries (eg, Bulgaria, Israel, the Netherlands, Poland). In 1,375 of 3,172 outbreaks (43%) where data were available, 50% were associated with farms, 22% with restaurants, 13% with private households, and 15% with various other forms of catering.[121]

Geographical Distribution

As a result of the changing and widening patterns of national and international food chains and increasingly mobile populations, many outbreaks of foodborne disease have become more diffuse in their geographical presentation. In addition, the development of the cold chain in food distribution and the widespread availability and use of commercial and domestic refrigerators and deepfreezes may distort the time scale of outbreaks, with cases appearing over a period of several weeks (or months) in different districts, regions, or countries. Furthermore, people affected in the outbreak(s) may have dispersed widely to other areas (or other countries) between eating contaminated food and the onset of illness several hours, days, or weeks later. In consequence, many outbreaks, in particular common-source outbreaks, may remain undetected unless attention is drawn to them via coordinated surveillance of increased reporting of the frequency of an unusual (serotype, phage type) strain.

Rural areas commonly have a higher reported incidence of campylobacter and VTEC than urban areas, reflecting the zoonotic and environmental aspects of many infections.

Contributory Factors

Factors contributing to outbreaks of foodborne disease may not be readily identifiable, particularly after a delay of several days (or longer) between food preparation and the beginning of the epidemiological investigation. In a study in England and Wales in the 1980s analyzing the factors that contributed to the occurrence of 1,479 food poisoning outbreaks, the most important factors reported were the preparation of food too far in advance (57%), storage at ambient temperatures (38%), inadequate cooling (32%), inadequate reheating (26%), eating raw foods (6%), and cross-contamination (6%).[133] In the US the most commonly reported practices that contributed to foodborne outbreaks during 1988 to 1992 were improper holding temperatures followed by poor personal hygiene of food handlers.[127]

Subjectivity on the part of the investigator reporting outbreaks may, however, be misleading, resulting in some factors being over-represented and others under-represented. Although difficult to assess objectively, cross-contamination between raw foods and precooked (or unheated) foods through faulty food hygiene practices (inadequately washed hands, contaminated surfaces, utensils) appears to be a particularly important transmission factor in salmonella and campylobacter infections. The role of infected food workers as the origin of foodborne disease is unclear other than in staphylococcal and some viral infections. In many instances, infected food workers are victims of the outbreak and not the source.

37.5.2 Recognition and Investigation of Outbreaks

Information relating to the occurrence of outbreaks usually is obtained by laboratory (hospital, public health, reference) or physician reporting, from neighboring health departments, and on occasion the media or from individuals affected. The successful investigation of any episode of suspected foodborne disease (outbreak or sporadic case) is facilitated by the physician or laboratory promptly reporting the incident (by telephone, fax, or electronically) to the appropriate health department (Figure 37–3). The flow of information can be further enhanced where a close working relationship exists among laboratory staff, primary care and public health physicians, EHOs, community nurses, and veterinary colleagues, particularly where such relationships have been developed before rather than during an outbreak.

Although in many instances the investigations of outbreaks are successful in identifying food vehicles and etiological agents, and in indicating where control measures should be applied, in other instances the results of investigations are inconclusive. Nevertheless, information thus obtained may provide useful epidemiological data available for subsequent outbreak investigations.

Many cases of foodborne disease occur as sporadic infections and the significance of a single case within the area of an individual physician or local health authority should never be minimized or ignored. In an increasing number of incidents, this could be the first case to be identified in a multidistrict or multistate outbreak, affecting persons widely scattered geographically who had eaten at the same restaurant, attended the same social function, or had eaten contaminated food purchased from different retail outlets (eg, shops, supermarkets) across the country.

Geographically scattered outbreaks are less likely to be detected than locally occurring outbreaks because they are more frequently dependent on subsequent laboratory reporting of specific pathogens, particularly where unusual serotype or phage type strains of *Salmonella* or VTEC have been identified by a reference laboratory. Antibiotic-resistance patterns (antibiograms), DNA fingerprinting, or other identification techniques can also be utilized epidemiologically in the recognition of outbreaks, which were not otherwise apparent.

Routine reports of clusters of acute gastroenteritis in the US in 1993 initiated epidemiological and laboratory investigations in collaboration with the CDC and led to the identification of multistate outbreaks of illness from a Norwalk-like virus associated with eating raw oysters.[46,80] A microbiological and epidemiological study of sporadic cases of listeriosis in the US during 1988 to 1990 demonstrated the presence of *Listeria monocytogenes* in at least one food item from the household refrigerators of 79 of 123 affected persons; of these, one third of the refrigerators contained food(s) that grew *L. monocytogenes* of the same strain as had been isolated from the corresponding patient.[109]

A 5-year review of *E. coli* O157 infection between 1987 and 1991 highlighted the apparent absence of community outbreaks in Alberta, Canada, compared with Scotland, where numerous outbreaks were reported.[182] To what extent such differences were due to the more closely integrated interdisciplinary surveillance program existing within Scotland involving laboratory, public health, and veterinary medicine, supplemented by the accessibility of a dedicated reference laboratory routinely receiving and typing isolates, is not certain.

Routine laboratory surveillance can be used to monitor current trends in the detection of emerging sero/phage types as well as for retrospective analyses. The availability of computer software technology in monitoring epidemiological data can readily detect increases in the number of reports relating to specific sero/phage types over and above normal expected levels.[48]

Following notification or receipt of a laboratory report, a visit is made usually to the home of the affected person by an EHO (or public health nurse) to initiate the epidemiological investigation. This practice may differ widely from country to country, even among health jurisdictions within individual countries, with much depending on national (or local) interests or priorities in investigating reported cases of foodborne disease. In addition to obtaining clinical, epidemiological, and sociological details of the patient (and his or her family), a detailed food history is important, recording what food was eaten (within the home and elsewhere) before the onset of illness by those affected within the relevant incubation period of the reported etiological agent. A good clinical history of the patient's illness is always important and may lead to the likely etiological agent, if not already known. When the predominant presenting symptom is vomiting, as in staphylococcal food poisoning, the incubation period is usually less than 6 hours, but it may be as long as 24 to 36 hours in viral gastroenteritis. If diarrhea is the presenting feature, an incubation period of 8 hours or more is likely.

When the incubation period is particularly short (15 minutes to 1 hour), a toxic or chemical etiology should be considered.

Laboratory investigations need to be undertaken concurrently with the epidemiological inquiries, with the appropriate laboratory alerted as early as possible in the outbreak to enable preparations to be made to deal with the extra workload anticipated. Samples of feces (or other clinical specimens) from cases, suspected foods, and, where available, swabs from kitchen surfaces and utensils (knives, spoons, mixing bowls) should be submitted to the laboratory as soon as possible. Specimens of vomitus are particularly valuable when staphylococcal, *Bacillus cereus*, viral (or chemical) foodborne disease is suspected. Blood samples may also be helpful in the diagnosis of botulism, VTEC infection, and associated HUS.

Assessment of the available clinical, laboratory, and epidemiological findings should help direct the investigation toward a particular meal or item(s) of food. In a point-source outbreak associated with a specific social function, the investigation can be limited to the one (or more) meal(s) common to all those affected. A food-preference study relating to foods that were consumed or were not consumed by those affected can be particularly helpful in pointing toward the contaminated food(s). The study of catering records (or menus) in restaurants, hospitals, and schools may be helpful in pinpointing suspect foods. The location of the food preparation areas and the juxtaposition of cooked foods and uncooked foods (eg, poultry or other raw meats) should be noted specifically, together with storage facilities (eg, refrigeration) and staff food-handling practices. Inquiries should also be made concerning the health (or recent illnesses) experienced by food-handling and catering staff, and the nature of their work in relation to the preparation and serving of implicated food (or meals). This may be particularly important in the investigation of salmonella, shigella, or viral outbreaks.

Other than in point-source outbreaks, it may be difficult to obtain a meaningful food history. Nevertheless, the patient and family should be asked about their regular (and also occasional) eating and shopping habits, with any unusual items noted. Individual food dislikes or dietary restrictions for personal, ethnic, or religious reasons should also be inquired about, enabling certain foods to be excluded from the investigation. In every investigation, it is important to keep an open mind as to the likely food vehicle. Unusual vehicles that have been featured in foodborne outbreaks have included bean sprouts,[102,110] chocolate,[34,59] marshmallows,[85] powdered milk,[9,89,138] salami sticks[31] (salmonellosis); turkey franks,[11,109] pâté,[93,98] coleslaw mix,[146] hot dogs[148] (listeriosis); apple juice,[6,14] fromage frais,[24] fermented sausage,[21] yogurt[97] (VTEC); cheese,[10] yogurt,[101] garlic[142] (botulism); coconut milk[165] (cholera); lettuce[77] (shigellosis); and soft fruits (viral hepatitis A).[8,113]

In the investigation of any outbreak, the cardinal features that should always be noted are:

- ***Time***—When did the illness start? Did all those affected fall ill within a few hours or so of each other, or were they affected over several days or more?
- ***Person***—Who were affected? What common features were present? Were those affected predominantly infants, children attending a day-care center, employees of the same firm, members of the same social club, or a particular ethnic or socio-economically disadvantaged group?
- ***Place***—Where did the incident occur? Was the outbreak associated with a particular hotel or canteen, takeout food from the same restaurant, a wedding party, or other social function?

Plotting an epidemic curve based on the dates of onset of illness of those persons affected in the outbreak should be undertaken. The epidemic curve as exemplified by three different outbreaks of *E. coli* O157 infection (Figures 37–4, 37–5, 37–6) is helpful in giving an indication to the investigator of the type of outbreak being dealt with:

- A ***point-source*** outbreak is associated with a single meal or event involving a specific social or other occasion (eg, wedding meal, christening party, school lunch, golf club outing). In point-source outbreaks, the times (minutes, hours, days, or weeks) of onset of illness (depending on whether the relevant infection has a short or long incubation period) are usually closely clustered together with any subsequent cases due to secondary person-to-person spread appearing later (Figure 37–4).
- A ***common-source*** outbreak involves different groups of persons eating, for example, at the same restaurant or canteen, possibly over a period of several days or associated with eating a contaminated food obtained from the same producer, wholesale, or retail outlet(s) over a period of days, weeks, or months, so that onset of ill-

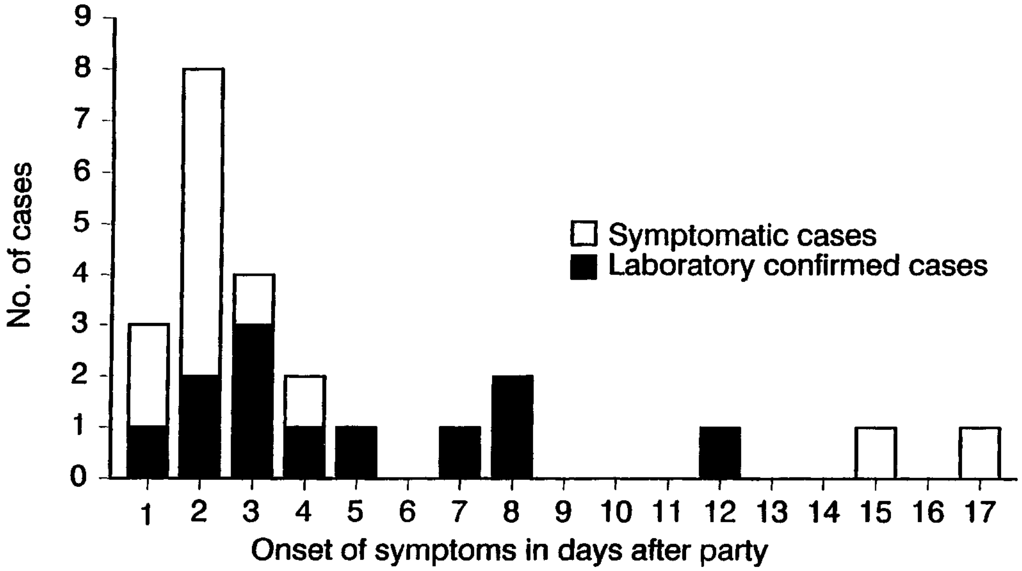

Figure 37–4 Dates of Onset of Illness in a Point-Source Outbreak of *E. coli* O157 Infection Following a Meal at a Party, June 1987. *Source:* Reprinted with the permission of Cambridge University Press from R.L. Salmon et al., A Christening Party Outbreak of Haemorrhagic Colitis and Haemolytic Uraemic Syndrome Associated with *Escherichia coli O157: H7*, *Epidemiology and Infection*, Vol. 103, pp. 249–254, © 1989, Cambridge University Press.

ness dates are more widely distributed (Figure 37–5).

- A ***person-to-person*** outbreak involves subsequent spread occurring in a hospital (nosocomial) or other residential institution or in the wider community, with the distribution of cases compatible with ongoing incubation periods (Figure 37–6).

The shape of the epidemic curve may be affected by variations in the range of the incubation period of the etiological agent responsible or, when occurring in the outside community, by the occurrence of missed (ie, unidentified) cases. In staphylococcal food poisoning, the incubation period may range over a few hours only; in salmonellosis or VTEC infection, it may range over a period of several days, most usually 2 to 3 but possibly longer, and in viral hepatitis over 4 to 6 weeks.

Interpretation of the epidemiological data obtained from the questioning of individual, and possibly numerous, cases in an outbreak investigation can be daunting. In recent years, this has been greatly facilitated by using modern computer programs such as Epi-Info, which can compile and quickly analyze the data.

37.5.3 Analytical Epidemiology

Analytical epidemiological studies such as cohort or case-control studies are frequently undertaken in the investigation of outbreaks, although the possibility of biased results should be guarded against and, where possible, the results should be supported by other (eg, microbiological) data.

Cohort Study

In a cohort study, a comparison is made of the food histories of those people who became ill and those people who remained well, following the consumption of a common meal or after attendance at a social function where food (eg, buffet meal) had been provided. The following are examples of cohort studies.

In the investigation of a point-source outbreak of *E. coli* O157 infection affecting guests at a christening party in England, a food history was taken from affected guests who were microbiologically positive and from others who were unaffected. Food-specific attack rates involving the two groups showed that clinical illness was associated with the consumption of turkey-roll sandwiches ($P < 0.001$), frankfurter sausages ($P < 0.025$), and pork pie ($P < 0.05$). Further analysis of consumption of sandwiches with pork pie or frankfurters demonstrated an independent, stronger association between turkey-roll sandwiches and illness ($P < 0.025$).[144]

An outbreak of cholera affected four people who had attended the same party in Maryland in August 1991 and was associated with the consumption of frozen coconut milk imported from Thailand. Epidemiological and laboratory investigations demon-

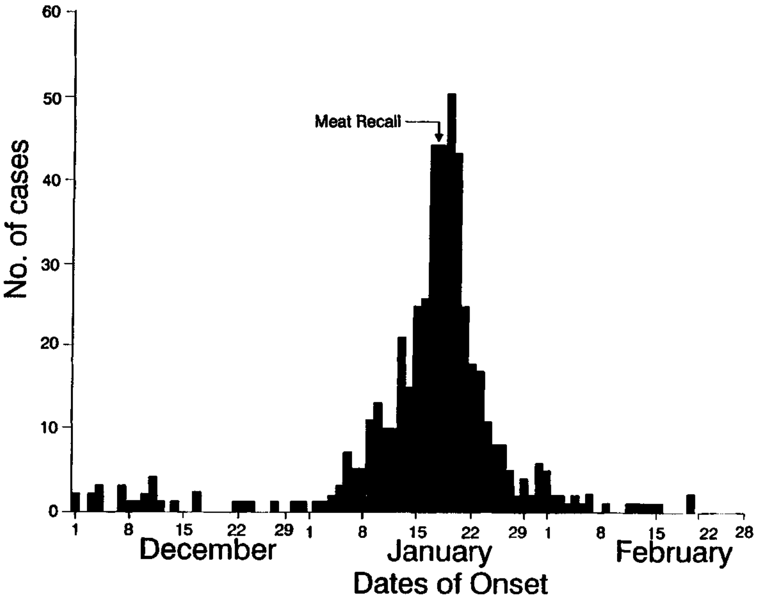

Figure 37–5 Dates of Onset of Illness in a Common-Source Outbreak of *E. coli* O157 Infection from a Multistate Distributed Food Product, 1992–1993. *Source:* Adapted with permission from B.P. Bell et al., A Multistate Outbreak of *Escherichia Coli* O157:H7—Associated Bloody Diarrhea and Hemolytic Uremic Syndrome from Hamburgers. The Washington Experience, *JAMA*, Vol. 272, pp. 1349–1353, © 1994, American Medical Association.

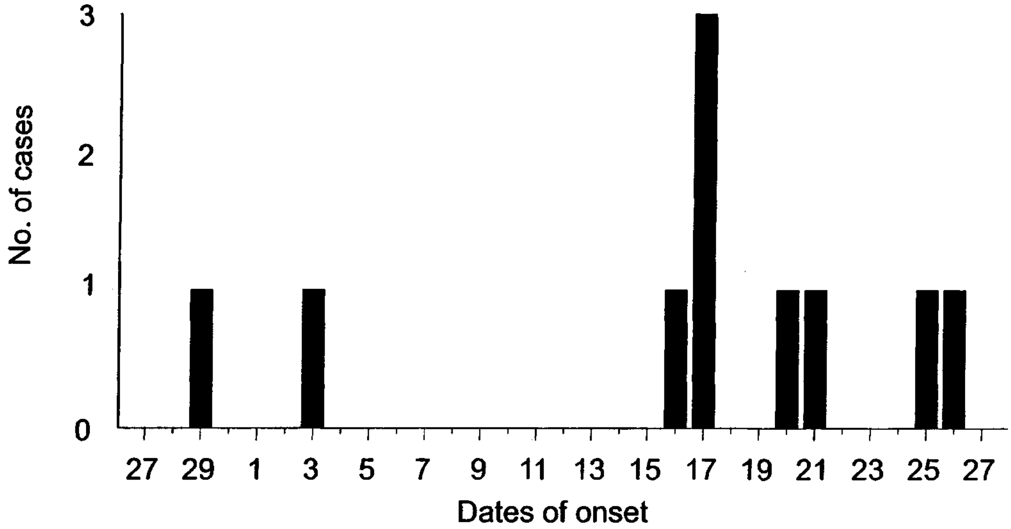

Figure 37–6 Dates of Onset of Illness in an Outbreak of *E. coli* O157 Infection due to Person-to-Person Spread in a Psychiatric Hospital, September-October 1990. *Source:* Adapted with permission from H.S. Kohli et al., A Severe Outbreak of *E. coli* O157 in Two Psychogeriatric Wards, *Journal of Public Health Medicine*, Vol. 16, pp. 11–15, © 1994, Oxford University Press.

strated that infection was associated with attendance at this party (A) and not with a second party (B) held later that same day at the same venue; 4 of 6 people who attended party A had evidence of *Vibrio cholerae* O1 (biotype El Tor, serotype Ogawa) infection compared with 0 of 15 who attended party B (P = 0.003). Subsequent investigation by the FDA resulted in the recovery of *V. cholerae* of the same type from 1 of 6 unopened bags of the same brand of coconut milk as had been consumed by those infected.[165]

Not every analytical study, however, is successful in providing the necessary epidemiological evidence. Following an outbreak of VTEC infection due to *E. coli* O111, in which 10 children developed HUS in northeast France in late 1992, two cohort studies were undertaken comparing the respective food histories of the 10 children who developed HUS with other children who had not developed HUS, and the 10 HUS children and 16 others who had gastroenteritis only with 62 well children at the school attended by the first group of cases. Neither study showed any association between food or water and the occurrence of illness. This, together with the evidence of the epidemic curve with onset of illnesses dates spread over a 14-week period, suggested possible person-to-person spread or spread from a continuing common, possibly food, source.[22]

Cohort studies also can be undertaken on a prospective basis by studying groups of persons (veterinarians, farmworkers, abattoir workers), who potentially are occupationally (or otherwise) exposed to specific enteropathogens.

Case-Control Study

In a case-control study, it is necessary to form an appropriate hypothesis (the null hypothesis) based on the results of earlier descriptive epidemiological findings relating to sociological and other features (eg, eating, shopping, work, or leisure activities) of those people affected that a particular event could not have been a significant factor in causing the outbreak.

In any case-control study, it is important to establish a strict definition of both cases and controls to be included in the study. It usually is necessary to recruit at least two controls (ie, unaffected people who potentially were exposed to the same risk of acquiring infection) for every one case, although in practice this may not always be possible. Controls may be nominated by the case (or parent) in the form of neighborhood controls, from the community health index, from other hospitalized people with an unrelated disease condition or through family physician practice registers, but in each instance controls must be matched with cases as far as possible by age, sex, and social characteristics. Interviewing cases and controls using a prepared, standardized questionnaire may be undertaken either on a face-to-face basis or by telephone, preferably by experienced interviewers. The following are examples showing case-control studies.

In the investigation of a nationwide community outbreak in the UK in 1988 due to *Salmonella typhimurium* definitive type (DT) 124, which had not been apparent as an outbreak at local level, initial epidemiological inquiries suggested that a particular brand of imported salami stick was the likely vehicle of infection. A case-control study showed a statistically significant association between eating imported salami sticks from West Germany and the onset of illness;[31] *S. typhimurium* DT 124 was isolated from a salami stick in an unopened packet obtained from the home of one of the cases interviewed. As a result, the manufacturers withdrew the product from sale.

Following an outbreak of *S. dublin* that affected 42 people in England and Wales in 1989, a case-control study showed a statistically significant association between infection and consumption of soft, unpasteurized cow's milk cheese imported from Ireland. *S. dublin* was subsequently isolated from cheeses obtained from the manufacturer. Control measures included withdrawing the cheese from retail sale, a press release, and a food hazard warning from the Department of Health. The manufacturer subsequently decided to pasteurize milk used in the production of cheese for the UK market.[91]

In September 1994, the Minnesota State Health Department detected an increase in the number of laboratory reports of *S. enteritidis* infection. A case-control study implicated a nationally distributed brand of ice cream associated with the outbreak, following which the product was recalled; *S. enteritidis* was isolated from 8 of 266 ice cream products examined.[65]

Case-control studies have been used on numerous other occasions in incriminating the food vehicle responsible for community outbreaks of other foodborne diseases. Among these were outbreaks of:

- botulism involving chopped garlic in soya bean oil in Canada[142]
- listeriosis involving foods such as brie cheese[60] and pork tongue in jelly[72] in France, Mexican-style cheeses in California,[87] coleslaw mix in Canada,[146] and pâté in the UK[93]
- paratyphoid fever associated with goat's milk cheese in France[40]
- shigellosis in northern Europe associated with imported lettuce from Spain[77]
- staphylococcal food poisoning in the US associated with imported canned mushrooms from China[83]
- VTEC infection associated with hamburgers,[13,36] heat-treated milk,[95,176] vegetables,[96] and yogurt[97] in the US and UK.

Case-control studies can also be used to:

- study emerging community infections, such as those due to *S. virchow* associated with the consumption of poultry meat[185]

- investigate methods of transmission of enteropathogens such as *Campylobacter jejuni*[39]
- study possible disease-associated clinical complications such as the association of campylobacter infection with Guillain-Barré syndrome[112]
- identify risk factors associated with sporadically occurring cases of campylobacteriosis,[1,103] listeriosis,[147] salmonellosis,[31,180] and *E. coli* O157 infection.[145]

Case-control studies are less likely to be successful in the investigation of common-source outbreaks involving commonly eaten foods (eg, poultry meat, hamburgers) than in outbreaks associated with less common foods (eg, salami, cheese), and their power should not be overestimated. Also it is necessary to be aware that analytical studies only identify food(s) consumed by those affected in the outbreaks, whereas the food may have been contaminated from another source (eg, raw meats) during preparation.

37.6 THE ROLE OF REFERENCE LABORATORIES

The contribution of reference laboratories in surveillance and in the recognition and investigation of foodborne outbreaks cannot be overemphasized. Reference laboratories for a wide range of specific foodborne pathogens (*Salmonella, Campylobacter,* VTEC, *Clostridium perfringens, C. botulinum, Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes*) have been established in many countries, in some instances having a primary role as the national surveillance center.

In addition to routine sero- and phage-typing, more definitive epidemiological information has been provided in recent years by the application of newer molecular technologies. These include plasmid profile and DNA fingerprinting studies by pulsed field gel electrophoresis (PFGE) demonstrating correlations, for example, between isolates of enteropathogens from patients and from specific food(s), such as imported poultry.[167] In other instances, discrimination has effectively been made between concurrently occurring foodborne disease incidents, which initially appeared to have been epidemiologically linked. While phage-typing remains a useful tool in routine surveillance and in the preliminary detection of common-source outbreaks, the newer technologies (including PFGE) provide a more definitive discrimination between individual microbiological strains.

The rise in the reported incidence of infection by *Salmonella enteritidis* in Europe (phage type 4) and in North America (phage types 8, 13, and 13a) observed during the 1980s was first detected by national salmonella surveillance programs based on routine phage-typing. Reference laboratory surveillance in the UK during the late 1980s and early 1990s similarly detected the emergence of antibiotic-resistant strains of *S. typhimurium*, in particular phage types 204c, DT 193, and DT 104. In other instances, community outbreaks of foodborne disease (salmonellosis, typhoid, VTEC, listeriosis) due to uncommon sero/phage-type strains have been identified by reference laboratory surveillance and the source of infection traced. Among the many salmonella outbreaks thus detected were those involving nationally or internationally distributed foods such as chocolate (*S. eastbourne, S. napoli*), salami sticks (*S. typhimurium* phage type DT 124), powdered milk (*S. ealing, S. anatum, S. tennessee, S. virchow*), peanut butter (*S. mbandaka*), savory snacks (*S. agona*), bean sprouts (*S. bovismorbificans, S. virchow, S. saint-paul*), cheddar cheese (*S. gold-coast*), goat's milk cheese (*S. paratyphi*), and ethnically prepared poultry-meat (*S. wangata*).

The regular forwarding of isolates of *E. coli* O157 by clinical laboratories to reference laboratories for further characterization similarly has been invaluable in identifying community outbreaks.[37,166] The availability of immunomagnetic separation (IMS) techniques improving the detection of *E. coli* O157 (and other enteropathogens), together with the use of PCR and verotoxin gene-probing from clinical, animal, and food sources, also have facilitated the investigation of outbreaks and the study of apparently unrelated sporadic cases. With the greater use of PCR and genetic probes for the detection of VT genes, it is anticipated that an increasing number of VTEC infections due to non-O157 serogroups will be identified in future years.

The role of reference laboratories in the investigation and control of foodborne diseases of zoonotic origin (eg, salmonellosis, campylobacter, VTEC) is further enhanced where human and veterinary surveillance programs are integrated. Routine typing of isolates of *Salmonella* submitted to the Scottish Salmonella Reference Laboratory indicated a possible epidemiological link implicating the role of seabirds feeding off sewage outfalls in transmitting infection, and a human case of *S. zanzibar* infection in the

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north of Scotland and subsequent isolates of the same uncommon serotype over a period of several months from (1) milk samples at a nearby dairy farm, (2) "sick" calves at other farms, and (3) abattoir effluents in the same district.[74]

37.7 THE ANALYSIS OF TRENDS AND INTRODUCTION OF CONTROL MEASURES

37.7.1 Trends in Foodborne Disease

Reports of foodborne disease have increased in most westernized countries. Heightened awareness, improved ascertainment, new laboratory technologies, more comprehensive reporting, and enhanced surveillance have undoubtedly contributed to the upward trends observed in recent years. Notifications of food poisoning in the UK, for example, had risen steadily prior to 1982, during which year 17,331 cases (31 per 100,000) were recorded. Thereafter a five-fold rise in notification reports occurred between 1982 and 1994 when 87,692 cases (150 per 100,000) were recorded,[122] in part attributable to the relatively recent inclusion of reports of campylobacter infection in food poisoning statistics. Reports of isolates of *Salmonella*, *Campylobacter*, and *E. coli* O157 increased between 2- and 40-fold over the same period. Similar rises in salmonella isolation rates were observed in other European countries. Campylobacter infections now exceed salmonella infections in Australasia, Canada, and in the UK, and continue to be reported with increasing frequency in continental Europe and in the US where active surveillance has been introduced.[149]

Nevertheless it appears that a real increase in the incidence of foodborne diseases, mainly those (eg, salmonella, campylobacter, VTEC, listeriosis) of animal or environmental origin, has occurred. To what extent this has been due to the many changes that have taken place in food production and processing, such as more intensive farming methods (factory farming), best illustrated by the poultry broiler industry in which tens of thousands of birds are mechanically slaughtered daily within individual processing plants, is not clear. These developments have taken place in parallel with changes in eating habits and other socioeconomic factors, in particular the expansion of mass catering, the greater number of women working outside the home, and the availability of an increasing range of convenience foods.

Conversely, with the exception of viral-associated disease, infections primarily of human origin (eg, shigellosis, typhoid, paratyphoid) that continue to be important public health problems in developing countries are uncommonly transmitted by contaminated food (or water) in westernized countries.

37.7.2 Salmonellosis

The increase in reports of infection due to serotypes (other than *Salmonella typhi* and *S. paratyphi*) has been particularly noticeable in Europe, with most countries reporting a two-fold increase between 1985 and 1992.[121] In the UK, 12,846 infections (23 per 100,000) were reported in 1981 compared with 33,600 (58 per 100,000) in 1994.[122] In North America, lower rates showing little change have been recorded since the early 1980s, particularly in the US, with annual rates varying between 13 and 22 per 100,000. In 1994, 37,506 infections (14 per 100,000) were reported in the US to CDC[126] compared with 7,441 (26 per 100,000) to LCDC.[120]

For many years *S. typhimurium* was the most commonly reported serotype causing human infection. During the mid-1980s, an increased incidence was observed in the frequency of infections due to *S. enteritidis* (in particular phage type 4), which became the predominant serotype in most European and South American countries.[137] In Europe in 1980, fewer than 10% of salmonella infections reported were due to *S. enteritidis*, but by 1992 this figure had increased to more than 50% in some countries and 90% in others.[121] Veterinary surveillance reports in the UK increasingly featured *S. enteritidis* phage type 4 in poultry broiler and egg-laying flocks, concurrent with the upsurge in cases of human infection, which in many instances were associated with eggs and egg products.[32,33,45] Infection due to *S. enteritidis* in Spain had increasingly been associated with egg and egg-based products, such as mayonnaise, since 1978[107] and in the UK in more recent years with eggs imported from other European countries.[38,156] Despite some decrease in reported incidence since 1992, *S. enteritidis* continues to predominate as the most commonly identified serotype in many countries, although not as yet in Australia or New Zealand.

In North America, a marked increase in infections due to *S. enteritidis* (phage types 8, 13, and 13a) was also observed in the later 1980s, initially in northeastern states of the US, associated with outbreaks involving hen eggs,[141] and subsequently in other states and Canada. Outbreaks continue to be associated with the consumption of raw eggs[84] and related products. In 1993 for the first time, eggborne outbreaks due to phage type 4 were reported.[19] Between 1988 (47% of outbreaks) and 1992 (75%), *S. enteritidis* was the most common cause of salmonella outbreaks reported to CDC,[127] and by 1995 had overtaken *S. typhimurium* as the most frequently isolated serotype.

Although now less predominant in many countries, infection by *S. typhimurium* continues to cause considerable human disease. Antibiotic-resistant strains of *S. typhimurium*, in particular phage types 204c, DT 193, and DT 104, have increasingly appeared in recent years in Europe[20,168,180] and North America.[70]

Poultryborne salmonellosis involving a wide range of serotypes continues to be a major feature in most westernized countries, with *S. virchow* and *S. hadar*, for example, also having developed antibiotic resistance in recent years.[56] Irradiation of poultry, the cost-effectiveness of which in controlling salmonellosis has been demonstrated,[189] has been introduced in the Netherlands and in France. Following the introduction of environmental control measures within the poultry broiler industry, however, there are encouraging indications from some countries (eg, UK, Scandinavia) that contamination rates are decreasing.

37.7.3 Campylobacter Enteritis

Since the 1980s, campylobacter (*C. jejuni, C. coli*) infections have exceeded salmonella in their reported incidence in countries with established national surveillance programs (eg, Australia, New Zealand, Canada, the Netherlands, the UK). In New Zealand, for example, national rates of more than 220 per 100,000 have been recorded in recent years, exceeding 300 per 100,000 in some provinces.[130]

In the UK, more than 48,000 cases (84 per 100,000) were reported in 1994[122] with rates exceeding 200 per 100,000 in some areas of Scotland. In 1998, more than 64,400 cases (115 per 100,000) were recorded in the UK. Surveillance studies in the Netherlands have indicated that *Campylobacter* accounts for 15% of acute gastrointestinal disease compared with 5% due to *Salmonella*. Elsewhere in Europe, campylobacter infections continue to be uncommonly reported compared with salmonellosis.[121]

In the US, the extent of under-reporting nationally has been highlighted by active surveillance studies such as FoodNet, which showed that where laboratories in selected areas were routinely screening for *Campylobacter*, isolation rates of up to 71 per 100,000 were recorded[16,124,149] compared with national rates of 5 to 6 per 100,000.[124,163]

The epidemiology of campylobacter infection is considerably less clear than that of salmonellosis. In part this is due to a relative absence of identifiable outbreaks compared with the preponderance of sporadic cases and to the fact that laboratory typing schemes for *Campylobacter* generally have been less available. Consequently, epidemiological investigations often are incomplete, and surveillance programs have been less well developed in most countries.

Despite an increased understanding of the ecology of campylobacters, control measures are relatively difficult to apply within the poultry broiler industry. Cross-infection and cross-contamination are major problems, particularly within rearing farms and processing plants with their high stocking densities, high throughput, and carcass contamination from spilled intestinal contents. Eradication of *Campylobacter* from animal or environmental reservoirs may not be possible, although control measures implemented within poultry farms, such as chlorination of drinking water supplies and the application of specific hygiene measures during the rearing period, have reportedly been successful in reducing the number of organisms present.[106,178] The financial costs to the poultry industry of introducing and maintaining effective preventive measures continue to outweigh market profitability. Irradiation of the finished raw product would be particularly effective as a control measure, but related costs and customer acceptance remain a problem in most countries.

37.7.4 Listeriosis

During the mid-1980s, increases in reports of infection by *Listeria monocytogenes* were observed in several European and North American countries. Extensive foodborne outbreaks associated with unpasteurized soft cheeses and coleslaw mix were reported from Switzerland,[15] the US,[87] and Canada.[146]

In the UK, an increasing number of reports of listeriosis were recorded during the later 1980s, reaching epidemic proportions by 1988 when more than 300 cases were reported, the majority of which were due to two previously uncommonly identified strains (serotypes 4bx and 4b, phage types 6 and 7). These were shown to be indistinguishable by serotyping, phage-typing, and DNA restriction analysis from the isolates recovered from a particular brand of epidemiologically implicated pâté.[93] Control measures and government food warnings in the summer of 1989 relating to pâté consumption by at-risk pregnant women and immunosuppressed individuals were followed by a decline in the number of cases. The incidence of reported infection returned thereafter to the pre-epidemic levels of less than 100 cases that were being recorded annually during the early 1980s.

In France, multiregion community outbreaks were reported in 1992,[72] 1993,[136] and 1995,[60] with microbiological and epidemiological case-control studies identifying pork tongue, pork pâté (rillettes), and raw milk brie cheese, respectively, as the responsible food vehicles. Recall of the incriminated foods in conjunction with improved sanitation and other production control measures resulted in each of the outbreaks being brought under control. In contrast, in 1994 a significant reduction was recorded in the incidence of sporadic cases of infection throughout France that had been attributed to the official recommendations issued to at-risk population groups, in particular pregnant women.[73]

In a 1993 outbreak of gastroenteritis in Italy following a private supper party, *L. monocytogenes* was isolated from blood cultures of 2 of 18 affected people and from various leftover food and kitchen environmental samples, all of which were indistinguishable from the human isolates. Epidemiological investigations identified rice salad as the responsible food vehicle.[143] In July 1994, a similar outbreak of gastroenteritis and fever affected 45 people who had attended a picnic in Illinois. A cohort study demonstrated illness among 45 of 60 people who had consumed chocolate milk (attack rate 75%) at the picnic compared with no illness (0%) among those who did not consume this item; isolates of *L. monocytogenes* from 11 of 45 people affected, from samples of chocolate milk and from a tank drain at the dairy were shown to be indistinguishable from each other.[35]

Active surveillance for sporadic listeriosis in the US was initiated in 1986 based on a widening aggregate population in nine different geographic areas of the country. In 1993, a review of the effectiveness of the intervention control measures introduced (ie, aggressive food monitoring policies, improvements in cleaning, sanitation and control of hygiene during food processing, and issuing advice to vulnerable consumers), showed that these measures were associated with a substantial reduction in the reported incidence of disease in all nine surveillance areas.[161]

37.7.5 Verotoxigenic *Escherichia coli* (VTEC) Infection

Verotoxin-producing *Escherichia coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC) were first described in 1977 in Canada,[81] in which country the highest national rates of infection (mainly *E. coli* O157) were reported throughout the 1980s, increasing from 25 cases in 1982 to a peak of 2,407 (8.9 per 100,000) in 1989. The highest provincial rates (16.0 per 100,000) were recorded in Alberta, where surveillance was particularly active.[182] In 1990, VTEC infection became notifiable throughout Canada. Since then, reported national rates have decreased to 3.5 per 100,000. Following nationwide media attention focused on "hamburger disease" and HUS, intensive health education measures were introduced, supplemented in 1995 by the Canadian Paediatric Society, which issued detailed advice on the importance of high standards of food hygiene and cooking of raw meats.

In the US, although numerous community outbreaks have been recorded since the early 1980s,[61,62,131] very few states (eg, Washington, Oregon, Minnesota) had surveillance programs for *E. coli* O157 infection. As a result, reported national infection rates in the US remained low and trends were less apparent than those observed in Canada. In the extensive West Coast hamburger outbreak in 1993,[13,36] more than 100 cases were recognized belatedly in Nevada, Idaho, and California, states which, unlike Washington, had no surveillance arrangements. In October 1996, a further multistate (California, Colorado, Washington) outbreak with associated cases in British Columbia was reported, in which unpasteurized apple juice was identified as the responsible food vehicle.[6]

National surveillance was formally introduced in the US in 1993 following a recommendation by the Council of State and Territorial Epidemiologists that clinical laboratories begin culturing all stools (bloody and nonbloody) for *E. coli* O157.[30] Within 3 months of enhancing surveillance, a Connecticut laboratory reported the isolation of *E. coli* O157 from four people who were part of a larger outbreak associated with two separate picnics, at both of which hamburgers from the same supplier were served.[135] In 1994 following an increase in case reports, a New Jersey Department of Health survey showed that the number of laboratories culturing all diarrheal specimens for *E. coli* O157 had increased from 2 (10%) to 18 (90%) over the previous 12 months.[50] A later survey in December 1994 of 129 laboratories across the US showed that 38 (29%) were then screening all stools for *E. coli* O157; 32 (25%) screened all bloody stools; and 35 (27%) never screened.[18]

In the UK, an increasing number of cases and outbreaks have been reported since 1990, during which time a greater number of laboratories were extending their screening policies, examining a wider range of fecal specimens other than bloody stools in children. The highest isolation rates have been recorded in Scotland[154] with between 242 (4.7 per 100,000) and 506 (9.9 per 100,000) cases recorded annually during 1994 and 1998. Elsewhere in the UK, isolation rates in England and Wales increased from 0.5 per 100,000 in 1990 to 2.0 per 100,000 in 1997, thereafter decreasing to 1.8 per 100,000 in 1998. Within Wales where active surveillance of *E. coli* O157 has been undertaken since 1990, with all publicly funded laboratories (national health service and PHLS) routinely screening stools from all cases of acute diarrhea,[145] isolation rates of 0.9 to 1.2 per 100,000 were recorded.[122]

In November and December 1996, a major foodborne outbreak associated with meat from a single supplier in central Scotland affected a known 496 cases, of which 272 were laboratory confirmed; 22 elderly people died. *Escherichia coli* O157 organisms of the same type (phage type 2, verotoxin type 2) and PFGE profile, indistinguishable from that of the epidemic strain, were isolated from leftover gravy (meat sauce), from a meal consumed at a church lunch, and from an unopened, vacuum-packed (2.7 kg) packet of cooked meat from the same butcher source.[129]

Whether the incidence of infection outside North America and the UK has been as low as would appear or whether it is the result of differences in ascertainment, in laboratory techniques and policies, or in national surveillance priorities, is not clear. Nevertheless, *E. coli* O157 (and other VTEC) infections have been reported with increasing frequency in recent years from numerous countries worldwide (eg, Australia, Belgium, Denmark, France, Germany, Ireland, Israel, Italy, the Netherlands, New Zealand, South Africa, Spain, Sweden). During 1996 and 1997, extensive foodborne outbreaks of infection due to *E. coli* O157 were reported in Japan; in the largest, several thousand school children and staff were affected.[63] Internationally, the highest rates of HUS have been reported in Argentina,[88] where the disease syndrome has been recognized for many years.

Relatively little is known about the epidemiology of HUS or of infection by VTEC serogroups other than O157. In most instances where non-O157 serogroups were identified, they usually were associated with outbreaks of HUS such as those reported from South Australia,[21] France,[22] and Italy.[23] HUS has been made notifiable in some states in the US and in Australia. Although not notifiable in Europe at present, sentinel surveillance has been established in Austria, France, Italy, and Switzerland. In the UK, HUS was one of several conditions voluntarily reportable during the 1980s to the Surveillance Unit of the Royal College of Paediatrics and Child Health. This arrangement was discontinued in 1988, but restored in 1997 as part of a collaborative active surveillance study of HUS in children involving pediatricians, pediatric nephrologists, microbiologists, and epidemiologists throughout the UK and Ireland.

In April 1997, the WHO convened a consultative meeting on the "Prevention and Control of EHEC Infections" in Geneva, attended by experts from 14 countries to explore the health hazards posed by recent outbreaks. Enhanced investigation and reporting were among the many recommendations adopted at this meeting, with particular emphasis on the establishment of surveillance for HUS whether or not individual countries had the necessary microbiological resources to undertake the surveillance of EHEC (VTEC) infection.[187]

37.7.6 Other Foodborne Infections

Staphylococcal Food Poisoning

Food poisoning due to enterotoxin-producing staphylococci is one of the most commonly reported foodborne diseases internationally, particularly in Latin America,[172] in much of continental Europe,[121] and to a lesser extent in North America.[170] In the UK, although no longer a major cause of foodborne disease compared with the 1950s and 1960s, outbreaks continue to be reported.[184] Incidence and trends are difficult to measure because many cases are unreported unless occurring as a recognizable outbreak.

Overall, the incidence of disease appears to be decreasing in most western European and North American countries, largely as a result of the application of proper temperature control (cooking, chilling, refrigeration) in the preparation of susceptible foods such as in cold buffet-type meals. Elsewhere incidence varies widely in different countries and regions largely dependent on cultural dietary habits and food preparation methods. International outbreaks due to contaminated lasagne imported from Italy[186] and canned mushrooms from China[83] have been reported.

Clostridium perfringens *Food Poisoning*

Food poisoning due to *C. perfringens* is a commonly reported cause of foodborne outbreaks in many European[121] and Latin American countries.[172] Many cases go unreported due to the relatively mild, short-lasting nature of the illness, with epidemiological data restricted to information obtained from the investigation of point-source outbreaks.

Surveillance of *C. perfringens* food poisoning in the UK is coordinated by the PHLS Food Hygiene Laboratory, based on microbiological material from feces and food samples submitted by public health and hospital laboratories. A downward trend in the frequency of reported outbreaks has been observed since the mid-1980s, in particular outbreaks in hospitals and other health care facilities,[26] where a problem existed for many years before the removal in 1987 of immunity of hospital catering from the requirements of UK food hygiene legislation.

Viral Infections

The increase in laboratory reports of viral gastroenteritis has largely been due to the increasing availability of the electron microscope and its use in the investigation of outbreaks. A 40-year review of molluscan shellfish-associated (cockles, mussels, oysters) disease in the UK was published in 1985 and showed a marked increase in reported outbreaks with an established viral etiology (small, round structured viruses [SRSV], hepatitis virus A), many of which were caused by eating raw oysters or inadequately cooked cockles.[155] Similar experiences have been reported in the US[46,80] and in other countries.

Despite education and official health warnings to draw attention to the hazards of eating shellfish harvested in sewage-polluted waters, outbreaks of disease continue to be reported.[90] Viral infections in particular are greatly under-reported, with numerous disease episodes being inconclusively investigated or recognized. Due to the relatively short-lasting clinical effects of most cases of viral gastroenteritis and the 4 to 6 weeks' incubation period of viral hepatitis A, a food (or food-handler) source may not be readily suspected other than in point-source outbreaks associated with a specific meal or social function.[113]

Botulism

Botulism continues to be an important public health problem in much of continental Europe and among indigenous communities in northern regions of Asia and North America. Despite the clinical severity of most cases, the true incidence of disease is difficult to assess. Epidemiological and microbiological confirmation of the food vehicle(s) involved is often unobtainable unless a recognizable outbreak occurs. In some instances, the illness may be uncharacteristically mild and slowly progressive with the diagnosis mistaken for other neurological conditions (eg, Guillain-Barré syndrome, myasthenia gravis, psychiatric disorders, stroke), compounded by instances where those people affected have dispersed over a wide geographic area.[101,142] Infant botulism was first recognized in the US in 1976; since then, cases have been reported periodically from North and South America, Europe, and Australia.[44]

Shigellosis

Although continuing to occur periodically in epidemic form through person-to-person or environmental transmission, most usually affecting young children, shigellosis appears infrequently as a foodborne disease in westernized countries.

In the summer of 1994, several northern European countries—in particular Norway, Sweden, and the UK—reported an increase of *Shigella sonnei* infection mainly affecting adults. Microbiological and epidemiological evidence, including case-control and food preference studies, identified iceberg lettuce of Spanish origin as the responsible food vehicle.[5,77] Among the factors contributing to the termination of the outbreak(s) were (1) the coincidental seasonal changes in the source of supply of lettuce in northern Europe, (2) legislative measures prevailing within Norway prohibiting the import of vegetables when local products become available, and (3) public health warnings relating to the preparation of lettuce and other vegetables to be consumed raw.

Typhoid/Paratyphoid

Foodborne transmission of typhoid and paratyphoid infection has become an uncommon occurrence in westernized countries, with most cases reported presenting as sporadic infections affecting travelers returning from visits to Third World countries. A recent exception was the multiregion outbreak of paratyphoid that affected 273 people in France between August and November 1993, caused by eating contaminated unpasteurized goat's milk cheese.[40]

Foodborne transmission, however, remains a major public health problem in developing countries where drinking water supplies are less likely to be effectively protected from human sewage or where a high proportion of food workers may be chronic excreters of *S. typhi* or *S. paratyphi*.

Yersiniosis

Relatively little is known about the epidemiology of yersiniosis, despite *Yersinia* species having been recognized as a foodborne pathogen since the 1970s. Although outbreaks involving foods such as raw and pasteurized milk products, tofu (soybean curd), and chitterlings (pig intestine) have been reported in some countries, most cases occur as sporadic infections. The true incidence is unknown, with infection most commonly continuing to be reported in countries with a specific research interest (eg, Australia, Belgium, the Netherlands, Denmark, the Baltic states). Few countries other than Australia, Belgium, and Germany have yersiniosis as a notifiable disease.

Pigs are a natural reservoir of infection, with evidence from Belgium[162] and other European countries identifying consumption of raw pork as a significant risk factor.

37.8 CONCLUDING REMARKS

Despite the implementation of a wide range of control measures such as education, food hygiene legislation, and the Hazard Analysis Critical Control Point (HACCP) concept, there appears to have been a real increase in the reported incidence of foodborne disease in recent decades, part of which undoubtedly can be attributed to greater awareness, improved detection methods, and better surveillance systems.

Political, economic, and social changes, in parallel with greater industrialization and intensification of farming, food production, and processing methods and other factors such as changing consumer demands, may have contributed to the on going occurrence of foodborne disease as a worldwide problem. The majority of food manufacturers have now achieved and maintain high standards of hygiene during production, processing, and distribution. Nevertheless, many problems remain, for example, within the poultry-broiler industry with its high throughput of birds. Some anxiety also exists in relation to the production of soft cheeses prepared from unpasteurized milk, in particular by smaller producers, and in relation to those catering businesses that persist in using raw egg in foods conducive to microbial survival and growth.

The basis of any successful surveillance system is to maintain the flow of information and the development of good professional relationships among primary care and public health physicians, microbiologists, epidemiologists, and field workers (EHOs, community nurses). Electronic reporting systems have undoubtedly improved the flow of epidemiological information within and between countries, enhancing surveillance, nationally and internationally. Most modern foodborne diseases are zoonotic in origin, and their surveillance and control require close collaboration with veterinarians and other professional colleagues within the food industry. Geographically scattered community outbreaks are often difficult to detect, especially so in surveillance programs that do not include the expertise of a reference laboratory.

It has been suggested[4] in relation to the multistate hamburger *E. coli* O157 outbreak in the western US in 1993, that despite its magnitude, the public health problem coincidentally prevailing within the states of Nevada, Idaho, and California might not have been recognized, but for the epidemiological link identified in Washington. The outbreak, however, also drew attention to the limitations of the passive surveillance arrangements within Washington, resulting in a delay of some weeks between the occurrence of the first cases and the start of investigations.

Whether or not mandatory reporting systems provide better data than voluntary reporting remains controversial, with circumstances varying from country to country. Cuba, with its comprehensive legislative requirements, has collected national data on foodborne disease more consistently than any other country.[172] Within Europe the highest salmonella isolation rates during the late 1980s and early 1990s were recorded in the former Eastern bloc countries, where mandatory reporting was also a major feature. To what extent these rates were due to more comprehensive reporting or to poorer standards of food hygiene is uncertain. In Western Europe, salmonella surveillance is generally well developed with reporting rates higher than in North America despite the fact that salmonellosis is not formally notifiable in the UK other than as part of the clinical syndrome of "food poisoning" and has been discontinued as a notifiable disease in the Netherlands since 1985. In some countries (eg, the

Netherlands, France), increasing reliance has been placed on data from physician "sentinel" voluntary systems complemented by routine laboratory reporting. Whichever reporting arrangements (mandatory or voluntary) are used, the goodwill and collaboration of all health care workers involved is of paramount importance in providing good quality data.

Syndrome reporting, although providing less definitive epidemiological information than laboratory data, nevertheless has an important role in providing early warning of an emerging outbreak. The reporting by physicians of HUS, for example, even where microbiological confirmation is lacking, nevertheless provides an invaluable indicator of the occurrence of VTEC infection in the community. In future years, clinically based syndrome reporting may become of increasing importance in alerting health authorities, particularly if physicians with financial budgets restrict the number of clinical samples sent for laboratory investigation.

A limiting factor for laboratory-based systems can be the considerable financial costs of routine diagnostic and reference laboratory microbiology, exclusive of the further costs of research and development of new typing and detection techniques. The increasing emphasis currently prevalent in many countries toward financially oriented hospital and primary care health services in parallel with the development of commercially based private laboratories, with consequent reduction in government funding of hospital and public health laboratories, is regrettable and almost certainly will be a retrograde step.

The importance of investigating single cases of foodborne disease, however, should not be underestimated. In France, for example, only outbreaks (and single cases of botulism) are investigated. In many other countries including the UK, some health authorities, due to resource problems, restrict their investigations to formally notified cases or to recognizable outbreaks only. In consequence, important epidemiological data concerning sporadic cases can be lost, not least due to delays in identifying "common-source" community outbreaks. National and international outbreaks usually have small beginnings, often presenting initially as geographically scattered, sporadically occurring cases.

Administrative problems in correlating data obtained from different collection systems (statutory notifications, laboratory reporting, etc.) need not present unacceptable difficulties. Although the correlation of physician notifications with laboratory reports can be readily achieved electronically, linking data between different reporting systems should not be a priority for surveillance. The Communicable Diseases Computer Network in France, based on sentinel physician data on non-notifiable disease conditions, appears to correlate well with formal notification and laboratory-based surveillance systems.

Much still needs to be learned about the ecology and routes of transmission of foodborne zoonotic disease, especially campylobacter, listeria, and VTEC infections. The extent to which these are due to foodborne, waterborne, animal contact, or environmental transmission remains unclear. Little is known about the extent and duration of microbial carriage in food animals (cattle, sheep, etc.). Inter-disciplinary surveillance, allied to collaborative microbiological and epidemiological research, clearly has an important role to play in this area. Surveillance programs for all types of VTEC infection, and in particular HUS, clearly need to be further developed.

It is not clear why the reported rate of *E. coli* O157 infection in Scotland is consistently higher than elsewhere in the UK, or why there should be a disproportionately greater number of outbreaks. It is only since 1994 that laboratories in Scotland have been routinely screening diarrheal feces for *E. coli* O157. Within Scotland regional differences also occur with significantly higher infection rates reported most years by laboratories based in the east of the country than the west.[154] Following the major outbreak of *E. coli* O157 in central Scotland in November and December 1996, wide-reaching recommendations were made by a UK government advisory group emphasizing among other aspects (eg, Research, Enforcement Measures, Outbreak Control) the need for greater priorities to be given to the practicalities, costs and benefits of improvements to the arrangements for surveillance, and data collection and analysis of foodborne disease.[129] In 1998 proposals were placed before parliament for setting up a Food Standards Agency in the UK with far-reaching responsibilities relating to food hygiene and prevention of foodborne disease.[94]

National surveillance programs are as yet insufficiently developed in many countries. In 1997 following the major outbreaks of *E. coli* O157, Japan initiated a nationwide foodborne disease surveillance program with particular emphasis on *Escherichia coli* O157 (and other VTEC serotypes), *Yersinia enterocolitica*, *Campylobacter jejuni* and *C. coli*, *Salmonella enteritidis*, and *Clostridium botulinum* as target organisms. Attention has been drawn to the need to improve surveillance and investigation of foodborne disease internationally.[164,172] Although foodborne disease is recognized by WHO as an important public health problem, the development of surveillance programs has largely been hindered by the lack of political will by many governments, and in consequence deprived of adequate funding.

The need for ongoing analyses of surveillance data, prompt epidemiological investigation, timely communication, close collaboration between epidemiologists and microbiologists, with the importance of advising colleagues in other countries of the distribution of potentially contaminated products at an early stage, cannot be overemphasized. The effectiveness of Salm-Net (now Enter-Net) in Europe is an outstanding example of internationally coordinated surveillance leading to recognition, investigation, and control of foodborne outbreaks.

Foodborne disease is preventable. Microbial contamination levels in raw foods can be reduced significantly by good hygienic practices and the application of effective preventive measures throughout the food chain (eg, by the use of the HACCP concept and the selective use of ionizing irradiation). While the final control measure is dependent on the individual food-handler's (commercial or domestic) understanding of the importance of maintaining high standards of food hygiene in the kitchen, total reliance should not be placed in this area.

Even where effective control measures have been introduced, there may be political pressures to remove them. Milkborne infection, for example, has largely been controlled in countries with legislation requiring the heat-treatment of milk for retail sale, in particular cow's milk. Yet a powerful politically oriented lobby exists in many countries in favor of promoting a market in natural, untreated, organically grown dairy produce.

Routine surveillance, however, cannot always provide definitive scientific evidence implicating specific food vehicles. In addition, the quality of the data on which many programs are based may be open to criticisms such as the inexperience and/or lack of objectivity of approach of investigators, the accuracy and comprehensiveness of their reports, and unseemly delays in the publication of analysis of the data. Surveillance programs, however, have demonstrated their value in providing the epidemiological data necessary for the introduction of intervention measures in the control of foodborne disease. Nevertheless, following the introduction of appropriate control measures, surveillance has to be maintained at a high level in order to monitor their continuing efficacy.

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38 The *Aeromonas hydrophila* Group

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38.1 INTRODUCTION

The *Aeromonas hydrophila* group (motile Aeromonads, mesophilic *Aeromonas*) has presented a challenge to clinical and food microbiologists since the bacterium first was isolated and described as the cause of septicemia in frogs. Although this organism occurs widely in the environment, especially in various water supplies (the name *hydrophila* from the Greek *hydro* and *philos* means water loving)[49,58,66,68] and, as a consequence of contact with water, in many fresh and processed foods, its exact role as a cause of gastroenteritis is still disputed. Its wide occurrence in water and many foods, its ability to grow readily at 5° C (a temperature previously thought adequate to restrict the growth of foodborne pathogens),[98] and the fact that many isolates possess factors associated with virulence suggest that the presence of *A. hydrophila* in foods should be of major concern to food safety microbiologists.[138]

38.2 TAXONOMY AND CHARACTERISTICS

For the early history and background of the bacteria now assigned to the genus *Aeromonas*, see Eddy,[33] Farmer et al.,[35] and Kulp and Borden.[73] Through the years and different editions of Bergey's Manual, bacteria now termed the *A. hydrophila* group were placed in the following genera: *Bacillus*, *Bacterium*, *Aerobacter*, *Proteus*, *Pseudomonas*, *Escherichia*, *Achromobacter*, *Flavobacterium*, *Vibrio*, and *Necromonas*.[35] The *A. hydrophila* group is currently in the family *Vibrionaceae* with the genus *Vibrio*, based on the characteristics of oxidase-positive and motility by polar flagella.[12] Differentiating characteristics of members of the family *Vibrionaceae* are shown in Table 38–1. The family was created to differentiate these organisms from the *Enterobacteriaceae* and no phylogenetic relationships were implied. In 1986, creation of the family *Aeromonadaceae* was proposed[28] based on examination of 16S ribosomal RNA content, 5S RNA sequences, and ribosomal RNA-DNA hybridization data of a large number of isolates. This evidence indicated that *Aeromonas* spp. possess an evolutionary history sufficiently different from the *Vibrio*-

Table 38–1 Differentiation of the Genus *Aeromonas* from Other Genera of the Family *Vibrionaceae*

Test	*Aeromonas*	*Vibrio*	*Plesiomonas*
Inhibition by 0/129			
10 μg	R[a]	D[b]	D
150 μg	R	S[c]	S
Na^+ required for growth or stimulates growth	–	+	–
Inositol (acid)	–	–	+
Ornithine[d] decarboxylase	–	D[b]	+
Production of exoenzymes			
amylase	+	–	–
gelatinase	+	+	–
lipase	+	–	–
mol% G + C of DNA	57–63	38–51	51
Growth on TCBS[e]	–	+	–

[a]R, resistant
[b]D, different biotypes
[c]S, sensitive
[d]*A. veronii* is positive
[e]TCBS, thiosulfate-citrate-bile-salt-sucrose agar

Source: Data from P. Baumann and R.H.W. Schubert, Family *Vibrionaceae Veron* 1965, 5245AL, in *Bergey's Manual of Systematic Bacteriology*, Vol. 1, N.R. Krieg and J.G. Holt, eds., pp. 516–517, © 1984, Williams & Wilkins, and S.W. Joseph and A. Carnahan, The Isolation, Identification, and Systematics of the Motile *Aeromonas* Species, *Annual Review of Fish Disease*, Vol. 4, pp. 315–343, © 1994, Elsevier Applied Science.

naceae to warrant removal from this family. The phylogenetic locus is intermediate between the families *Enterobacteriaceae* and *Vibrionaceae*, and the phylogenetic depth of the genus *Aeromonas* suggests that *Aeromonas* spp. represent a discrete family of eubacteria. This proposed separation is pending.

Organisms in the genus *Aeromonas* are separated generally into two groups based on physiological traits[116] and genetic analyses.[80,117] The genetic analyses indicated that the genus *Aeromonas* consisted of two genotypically legitimate groups, a diverse group of motile aeromonads (designated as species *Aeromonas hydrophila*) and a genetically more homogeneous group of non-motile aeromonads comprising *Aeromonas salmonicida*.

In addition to being non-motile, the *A. salmonicida* group does not grow at 37° C (optimum at 10-15° C) and is sometimes described as psychrophilic. These organisms are found on, and cause disease in, salmid fish. They produce a characteristic brown pigment, especially on media containing tyrosine or phenylalanine.

The motile aeromonads are facultatively anaerobic, Gram-negative rods 1.1 to 4.4 μm long and 0.4 to 1.0 μm wide. Most strains possess polar monotrichous flagella, although some produce short lateral flagella and a few produce lophotrichous flagella. The motile *Aeromonas* species do not produce pigments. Colonies on nutrient agar are translucent to white or buff in color and are round and raised with an entire edge and a smooth surface. The aeromonads are heterotrophic and ferment carbohydrates with production of acid or acid and gas. Biochemically, they are similar to the *Enterobacteriaceae*; however, they can be easily differentiated from the *Enterobacteriaceae* by their ability to produce oxidase. They also produce catalase and reduce nitrates to nitrites. All *Aeromonas* species other than *A. salmonicida* are mesophilic and grow from 4-5° to 42-43° C, with an optimum growth temperature of 28° C. In brain heart infusion broth, the growth range at 28° C is between pH 4.5 and 9.0 and NaCl concentrations of 0% to 4%. Aeromonads are not susceptible to vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine).[116]

The classification of the *A. hydrophila* group has been confusing and has changed considerably over time. The introduction of DNA hybridization techniques into bacterial taxonomy allowed bacteria to be classified on the basis of their genetic relationships. After genetic relationships are established and bacterial groups separated, bacterial taxonomists then locate phenotypic traits to permit the routine differentiation of genotypes. Such is the case with the *A. hydrophila* group.

At present, there are 14 recognized DNA hybridization groups (HGs) of *Aeromonas*, which comprise 14 genospecies and 10 phenospecies, with 4 unnamed.[56] A scheme has been developed based on nine biochemical tests and two antibiotic sensitivities that will allow separation of the currently known HGs (Table 38–2). Because the scheme has been developed only recently, most isolates from food have been reported and described as *A. hydrophila* group, motile *Aeromonas*, or have been classified by the older biochemical scheme of Popoff et al.[117] as *A. hydrophila, A. sobria,* and/or *A. caviae.*

Two recent food surveys have classified isolates beyond these three species. In the first, using an expanded biochemical scheme, *A. schubertii* was identified from Brazilian white cheese.[136] In the second, using DNA hybridization techniques, *A. jandaei* and *A. veronii* biotype *sobria* were identified in addition to these three species.[152]

38.3 EPIDEMIOLOGY AND CHARACTERISTICS OF THE DISEASE

The role of the *A. hydrophila* group as a disease agent has been recognized since its initial isolation as the cause of red leg disease in frogs.[124] It remains an important cause of disease in fish.[8] In humans, *Aeromonas* spp. are associated with two distinct disease syndromes: septicemia and various tissue infections (*A. veronii* subsp. sobria and *A. hydrophila*)[54,85] and gastroenteritis (any mesophilic *Aeromonas* spp. but principally *A. hydrophila* and *A. veronii*).[85]

Although the one feeding study of *Aeromonas* failed to demonstrate diarrhea in human volunteers[89] and there is currently not a suitable animal model for diarrhea, a compelling argument can be developed to support the notion that the mesophilic *Aeromonas* group can cause human gastroenteritis (a less rigorous argument for the consideration of *A. hydrophila* as an enteric pathogen was presented by Ljungh and Wadstrom in 1985[78]).

The following facts indicate that *Aeromonas* spp. may be a cause of gastroenteritis in humans:

- Food isolates of the *A. hydrophila* group possess various factors associated with virulence in other enteric pathogens including various enterotoxins, hemolysins, proteases, hemagglutinins, and endotoxins.[1,94,97,104,119,120]
- The *A. hydrophila* group is isolated with greater frequency as the sole pathogen from certain individuals with diarrhea than other enteric pathogens.[3,37,40,49] This increased frequency was observed in children[3,40] and adults.[37] The three groups of investigators[3,37,49] noted a seasonal variation, with higher isolation rates seen during the warmer summer months. Hazen et al.[47] observed higher *Aeromonas* levels in water during the warmer summer months, suggesting a link to human illness; higher levels in water could lead to a greater chance of contamination of food or the possibility that water itself is the vehicle for these bacteria.
- *A. hydrophila* has been isolated as the sole or primary pathogen from food implicated in outbreaks of gastroenteritis.[2,5,67] In the first, Abeyta et al.[2] isolated *A. hydrophila* from oysters associated with a large foodborne disease outbreak. The bacterium was positive in the suckling mouse test and the Adrenal Y-1 mouse test, expressed hemolysin, and showed activity in the rabbit ileal loop (RIL). The isolates came from oysters that had been frozen for 1½ years, and there were no patient isolates available for comparison. In the second, *A. hydrophila* with identical biochemical and ribosomal RNA gene restriction patterns was isolated from both the patient with diarrhea and the leftover food.[5] The last outbreak was associated with a shrimp salad. *A. hydrophila* was detected in salad samples at levels ranging from 10^6 to $>10^7$ cfu g^{-1} of food. Other common foodborne pathogens were either absent or detected at low levels. The isolates from this study produced the following virulence-associated factors: β-hemolysin, cytotoxins, cytotonic toxins, and enterotoxins and they adhered to and invaded Henle 407 cells.[67]
- The *A. hydrophila* group is associated with infections in patients immunocompromised by treatment for various malignancies.[32,45,135] These infections include both systemic bacteremias[32,45] and diarrhea.[135] In retrospective patient interviews, Sherlock et al.[135] determined that their patients had no recent contact with water or fish, suggesting that their gastrointestinal tract was the likely source of the bacterium.

Table 38–2 Phenotypic Tests To Identify Currently Recognized *Aeromonas* Hybridization Groups (DNA HGs)

		Test Results										
DNA HG	*Phenospecies*	*Esculin hydrolysis*	*Gas/ Glucose*	*Ampicillin Resistance*	*Acid from Arabinose*	*Lysine Decarboxylase*	*Cephalothin Resistance*	*Ornithine Decarboxylase*	*Acid from Sucrose*	*Voges-Proskauer*	*Acid from Mannitol*	*Indole Production*
HG 1	*A. hydrophila*	+	+	+	±	+	+	–	+	+	+	+
HG 2/3[a]	*A. hydrophila*	+	+	+	+	–	+	–	+	+	+	+
HG 4/5a[a]	*A. caviae*	+	+	+	+	–	+	–	+	–	+	+
HG 5b	*A. media*	+	–	–	+	–	±	–	+	–	+	±
HG 6	*A. eucrenophila*	–	–	–	+	–	–	–	–	–	+	+
HG 7	*A. sobria*	–	±	±	+	–	–	–	+	–	+	+
HG 8	*A. veronii* bv sobria	–	+	+	–	+	+	–	+	–	+	+
HG 8	*A. veronii* bv sobria	–	+	+	–	+	–	–	+	+	+	+
HG 9	*A. jandaei*	–	+	+	–	+	+	–	–	+	+	+
HG 10	*A. veronii* bv veronii	+	+	+	–	+	–	±	–	+	+	+
HG 11[a]	*Aeromonas* sp.	–	–	–	+	–	+	+	+	–	+	+
HG 12	*A. schubertii*	–	–	±	–	+	–	–	–	–	–	–
HG 13[a]	*A.* spp. gr 501	–	+	+	–	–	+	–	–	+	–	+
HG 14	*A. trota*	–	–	–	–	+	+	–	±	–	+	+

[a] HGs 2, 5a, 11, and 13 are not yet named; results based on incubation at 37° C.

Source: Reprinted with permission from S.W. Joseph and A. Carnahan, The Isolation, Identification, and Systematics of the Motile *Aeromonas* Species, *Annual Review of Fish Diseases*, Vol. 4, pp. 315–343, © 1994, Elsevier Applied Science.

- Serological evidence can provide further support for the causal relationship of the *A. hydrophila* group to diarrhea. Based on very few patients, neutralizing antibodies to various exotoxins (hemolysin, enterotoxin, and cytolysin) produced by the patients' isolates have been detected in patients' sera.[22,43,70] The significance of these observations is not known, especially since the report of Gurwith et al.[43] in which the maximum titer was detected about 2 months after the diarrhea ceased. This area, like many others in *A. hydrophila* research, awaits further clarification.

Patients with *Aeromonas*-associated gastroenteritis can present with several types of diarrhea. These include (listed in decreasing frequency): secretory, dysenteric, chronic, choleraic, and Travelers'.[54] The different types of diarrhea likely reflect virulence factors expressed by the different strains isolated from these cases of gastroenteritis. For example, Gracey et al.[40] indicated that their isolates produced enterotoxins and their patients presented with secretory-type diarrhea.

38.4 MECHANISMS OF PATHOGENICITY

Bacteria of the *A. hydrophila* group produce a wide range of factors associated with virulence in other bacteria.[17,54] These include structural components and extracellular (exotoxins) and cell-associated factors. Structural components, identified in various isolates of the *A. hydrophila* group, that may be related to virulence include pili, S-layer (the major virulence factor in *A. salmonicida*), lipopolysaccharides, outer membrane proteins, and flagella.[53] There has been, however, little research that demonstrates a relationship between these components and virulence in this group of bacteria. Data from the literature suggest that the various exotoxins most likely represent significant virulence factors in these bacteria. Bacteria of the *A. hydrophila* group produce a wide range of extracellular products including hemolysins (aerolysin), enterotoxins, a cholera-toxin cross reactive factor, a cytotonic enterotoxin, proteases, and siderophores. Cell-associated factors that may be related to virulence in these bacteria are invasins, serum resistance, and adherence factor(s).

Stelma et al.[142] proposed that the major, if not sole, virulence factor in *A. hydrophila* was β-hemolysin. They observed that β-hemolysin correlated with fluid accumulation in the RIL assay (used to detect enterotoxin). Palumbo,[101] in reviewing the literature on the formation of putative virulence factors by food and water isolates of the *A. hydrophila* group, observed that, based on 12 published studies, most isolates formed a series of factors including β-hemolysin. Strains of *A. hydrophila* and *A. sobria* produced enterotoxin (detected by the suckling mouse test) and hemolysin (rabbit red blood cells) in a meat extract broth held at both 12 and 5° C.[82] Temperature can influence the expression of various virulence-associated factors and, in many instances, there was greater expression at lower incubation temperatures.[100]

Most of the research concerning *Aeromonas* pathogenicity has focused on the putative enterotoxins produced by diarrheagenic strains. Despite the fact that this research has been ongoing for over a decade, little is known about the roles of these toxins in human diarrhea. Furthermore, information on the number and nature of the *Aeromonas* enterotoxins is both confusing and controversial. Investigators who have found evidence for one type of enterotoxin frequently have failed to find any evidence for the others.

The first reported *A. hydrophila* enterotoxin was a cytonic enterotoxin, similar to cholera toxin with respect to its activities, which included causing fluid accumulation in the RIL, rounding of Y-1 adrenal cells without death, and elevation of cyclic AMP levels in Y-1 cells. The purified toxin had a molecular weight of 15,000, was heat-stable at 56° C for 20 minutes, and was not serologically related to cholera toxin (CT).[76,77,79] Two hemolysins (alpha and beta) were purified, neither of which had enterotoxic activities in animal models.[78,80] Further evidence for a heat-stable *Aeromonas* cytotonic enterotoxin was obtained when the gene for *A. hydrophila* cytotonic enterotoxin was cloned into *E. coli.* The clone produced an enterotoxin that caused elongation of Chinese hamster ovary (CHO) cells and fluid accumulation in the RIL. Both of these activities were heat-stable at 56° C for 20 minutes, and neither activity was neutralized by antiserum produced against CT.[21]

Several other reports provide evidence for *Aeromonas* cytotonic enterotoxins, some quite different from the one described originally. These include: (1) a heat-stable (56° C, 20 minutes) cytotonic enterotoxin that cross-reacted serologically with CT and had biological activities, including causing fluid accumulation in the RIL, that were neutralized by anti-CT;[118] (2) a similar toxin that caused elevation of cAMP levels in CHO cells but was only partially neutralized by anti-CT;[131] and (3) a cytotonic enterotoxin with a molecular weight of 44,000 that was heat-labile at 56° C, free of hemolytic and cytoxic activities, and not cross-reactive with antiserum to CT.[23] The purified toxin caused fluid accumulation in the RIL, elongation of CHO cells, and elevation of cAMP levels in CHO cells. Recently another[84] novel cytotonic toxin with no homology to any other known microbial toxin was purified. This toxin caused fluid accumulation in infant mice and CHO cell elongation but did not elevate levels of cAMP in CHO cells. CHO cell activity was not neutralized by anti-CT.

Preliminary evidence for production of a cytotoxic enterotoxin by *Aeromonas* spp. was provided by several studies in which enterotoxin production was correlated with cytotoxic activity, ie, rounding and death of tissue culture cells. Cumberbatch et al.[31] correlated cytotoxic and RIL activities; all of their cytotoxic isolates were also hemolytic. Turnbull et al.[146] and Burke et al.[15] also noted strong correlations between enterotoxin production and hemolytic activity.

Asao et al.[6] provided the first direct evidence for a cytotoxic *A. hydrophila* enterotoxin when they purified a hemolysin from *A. hydrophila* strain AH-1 to electrophoretic homogeneity and showed that it caused fluid accumulation in the RIL and in suckling mice and was cytotoxic to Vero cells. The purified hemolysin had a molecular weight of 50,000 and was heat-labile at 56° C. The purified hemolysin from AH-1 was shown to be a β hemolysin, and antiserum to the purified hemolysin completely neutralized the RIL activities of filtrates of β-hemolytic *Aeromonas* isolates, indicating that the hemolysin alone can cause the changes in intestinal permeability associated with diarrhea. A polyclonal antiserum against purified AH-1 hemolysin cross-reacted with hemolysins produced by *Aeromonas* isolates from diverse geographic origins.[142] Several studies provided evidence that the *Aeromonas* β-hemolysins are a family of molecules related to the "aerolysin" originally described by Bernheimer and Avigad[13] and that these toxins occurred across species. The hemolysin produced by an *A. hydrophila* isolate (CA-11) from a United States (US) gulf coast oyster was immunologically related to the hemolysin produced by the Japanese strain, AH-1.[7] The CA-11 hemolysin also possessed unique antigenic determinants, indicating that the two toxins are related but not identical. Hemolysins from *A. sobria* and *A. veronii* were shown also to have enterotoxic activities and to be serologically related to AH-1 hemolysin.[24,66,86,143] Rose et al.[122,123] purified a 52,000 molecular weight protein that was hemolytic, cytotoxic, and caused fluid accumulation in the RIL. Their toxin cross-reacted with CT as well as with AH-1 hemolysin, but none of the toxin's biological activities were neutralized by anti-CT. Later studies showed that the CT cross-reactivity was due to small stretches of amino acid similarity between the two toxins and that there were also some homologous sequences with *Clostridium perfringens* A-toxin and listeriolysin.[25]

The results of several surveys suggest either that the cytotoxic enterotoxins are the most common *Aeromonas* enterotoxins or that their activities are higher than, or mask, those of the other toxins. Seidler et al.[133] found cytotonic activity in the filtrates of only 20 of 330 isolates, and Stelma et al.[142] found cytotonic activity in only 1 of 24 filtrates. Cumberbatch et al.[31] observed no cytotonic activity in the filtrates of 96 isolates, and Johnson and Lior[55] found no cytotonic activity in the filtrates of 73 isolates.

Some of the conflicting data generated in early studies, including the inability of researchers working with one toxin to detect the others, are becoming understandable as more studies are performed. Ljungh et al.[76,79] reported that purified β-hemolysin was not active in the RIL. This may have been due to the purification method they used. Their six-step procedure resulted in the recovery of only 0.6% of the original hemolytic activity; whereas the two-step procedure of Asao et al.[6] resulted in the recovery of 65% of the original hemolytic activity and probably yielded a product closer to the native molecule in all its properties. There may be separate loci on the hemolysin molecule for different biological activities, with the hemolysins produced by some isolates having the enterotoxic site masked.[25] This would explain the absence of a perfect correlation between hemolysin production and enterotoxigenicity among *Aeromonas* isolates, as well as the lack of RIL activity in the hemolysin purified by Ljungh et al.[76,79] The failure of many investigators to observe the activity of heat-stable cytonic enterotoxins could be due to insufficient amounts in crude filtrates of many isolates. This possibility was shown by Chopra et al.,[26] who cloned a heat-stable enterotoxin gene into *Escherichia coli* from an *Aeromonas* isolate that produced toxin levels too low to allow detection in culture filtrates. The inability of many researchers to detect the various heat-labile cytotonic enterotoxins is due probably to interference by the cytotoxins.

A. caviae is isolated usually from diarrheal stools of very young children or elderly adults.[4,60,70,72,91] The symptoms associated with *A. caviae* are reported to be less acute than those associated with *A. hydrophila* or *A. sobria*.[72] *A. caviae* generally is considered to have low pathogenic potential and to be nonenterotoxigenic. In one survey, 95% of 284 *A. caviae* isolates were nonhemolytic.[88] In other surveys, only 2 of 284 *A. caviae* isolates were enterotoxigenic, as measured by the suckling mouse test,[6] and only one cytotoxic and no hemolytic strains were found among 106 stool isolates.[71] Only one report provides evidence for a possible enterotoxin. Like earlier investigators, Namdari and Bottone[91] were unable to detect enterotoxin in filtrates of *A. caviae* grown in single-strength tryptic soy broth (TSB). However, when they grew the same isolates in double-strength TSB, they detected a heat-stable cytotoxin that also was enterotoxic, as demonstrated by the suckling mouse assay.

The mostly negative results in a human feeding study suggest that factors other than enterotoxin are important in *Aeromonas* enteropathogenicity. Fifty-seven volunteers were given five known RIL-positive enterotoxigenic strains, but only two volunteers developed diarrhea. These strains were not invasive, and it was hypothesized that they also lacked the necessary adhesion factors to colonize the intestine.[89] This hypothesis was supported by an earlier study showing that both enterotoxins and adhesins were necessary to produce diarrhea in volunteers fed orally with enterotoxigenic *E. coli*[129] and by later studies showing that few strains of *A. hydrophila* and *A. sobria* possess adhesions.[93]

Although the adherence properties of the motile aeromonads are not well characterized, some important information has been acquired. Aggregative adherence, similar to that of enteroadherent-aggregative *E. coli*, was observed in *A. hydrophila* and *A. sobria*. In contrast, *A. caviae* showed a diffuse adherence pattern.[92] Attachment to adrenal cells was correlated with the presence of pili and with specific *Aeromonas* spp.[27] Hydrophobicity and the ability to autoagglutinate did not correlate. *A. sobria*, *A. hydrophila*, and *A. veronii* showed a greater ability to attach than did *A. caviae*. Hokama et al.[48] described two types of pili on an *A. hydrophila* isolate, one rigid and straight (R-pili) and the other wavy and flexible (W-pili, fimbriae). Purified W-pili blocked adherence of the organisms to rabbit intestines, suggesting that the W-pili are a colonization factor. Thornley et al.[145] observed that clinical strains of *A. caviae* adhered to two human cell lines to varying degrees and noted a correlation between adherence and both piliation and hemagglutination. Nishikawa et al.[93] showed by electron microscopy that strains of *Aeromonas* spp. did not possess fimbriae, but these strains could invade Caco-2 cells. They concluded that these strains of *Aeromonas* spp. adhered to and invaded tissue culture cells by a mechanism that remained to be elucidated. One of the most interesting observations was that environmental enterotoxigenic isolates possessed numerous pili that were lost when infection was established, suggesting that environmental isolates may be more infective than clinical isolates.[63]

Another interesting observation was a negative correlation between adherence and the presence of an R-plasmid.[44] Some *Aeromonas* strains are capable of invading mammalian cells. In one study, 18 of 69 (26%) fecal isolates were able to invade HEp-2 cells; 13 of these strains were also enterotoxigenic. A relationship between invasiveness and biotype was noted also; 16 of the invasive strains were *A. sobria*, two were *A. hydrophila*, and none were *A. caviae*. The investigators suggested that the invasive strains produced dysenteric symptoms.[150] Another study demonstrated that some *Aeromonas* strains invaded the intestinal mucosa of rabbits.[115] The invasiveness of *A. caviae* was investigated using criteria developed earlier for *Campylobacter*. The results agreed with those of previous investigations in that the majority of *A. caviae* strains showed little or no invasive ability. However, 8% of the strains were invasive in HEp-2 cells and 25% were invasive in the more sensitive Caco-2 cell model.[134]

38.5 INCIDENCE OF THE BACTERIA IN THE ENVIRONMENT AND FOODS

As indicated in the Introduction, the *A. hydrophila* group occurs widely in the aquatic environment and its presence in food likely reflects contact of contaminated water with the various food products. The results of some incidence surveys of different water supplies are shown in Table 38–3. The reported incidence varied from detection in 33% of samples in one municipal supply to two instances with 100% incidence. The levels encountered range from 0.01 cfu ml^{-1} (the low limit of detection) up to 9×10^3 cfu ml^{-1}.

Bacteria of the *A. hydrophila* group possess two traits that aid aquatic bacteria in their contamination of food products: their ability to survive for long periods in various types of water[106] and their ability to grow in the presence of low levels of organic compounds.[106,147]

The isolation of bacteria of the *A. hydrophila* group from chlorinated drinking water[16] suggests

Table 38–3 Incidence of Members of the *A. hydrophila* Group in Different Water Supplies.

Source	*Bacteria Identified*	*Incidence, Number +/Total Number (%)*	*Level*	*Reference*
River water (India)	*Ah**	(100%)	0.043 cfu ml^{-1} to 10^{2} ml^{-1}	114
Municipal water (Sweden)	*A. hydrophila, A. sobria*	23/27 (85%)	0.01 cfu ml^{-1} to 8.6 cfu ml^{-1}	68
Municipal water (European city)	*Aeromonas* species	78/239 (33%)	<0.04 cfu ml^{-1}	83
Lake water (US)	*Ah**	135/147 (92%)	0.1 cfu ml^{-1} to 9 × 10^{3} cfu ml^{-1}	47
Estuary (Chesapeake Bay)				
water	*Ah**	(100%)	<0.3 cfu l^{-1} to 5 × 10^{3} cfu ml^{-1}	58
sediment	*Ah**		*ca* 4.6 × 10^{2} g^{-1}	

*Ah** indicates biochemical testing not done to speciate isolates beyond the *A. hydrophila* group.

that these bacteria may be resistant to the levels of residual chlorine found in potable (drinking) water. However, Knochel[64] demonstrated that *A. hydrophila* was more chlorine-sensitive than coliform and other indicator bacteria and that any level of chlorine that inactivated coliforms would also inactivate *A. hydrophila*. The presence of *A. hydrophila* in properly chlorinated water may be explained by a high organic content, which would react with the residual chlorine, or by the presence of the bacterium in a protective biofilm inside the distribution system or recontamination by the *A. hydrophila* group.

The *A. hydrophila* group has been isolated from virtually every type of food, including fish (Table 38–4), dairy products (Table 38–5), poultry (Table 38–6), red meats (Table 38–7), and miscellaneous foods (Table 38–8). Water is the likely source of these bacteria in most foods, especially because animal feces contain only low levels of them. The levels encountered in foods vary widely, from <10^{2} CFU g^{-1} to 10^{7} CFU g^{-1}. The counts listed in Tables 38–4 to 38–8 are for the products at the time of purchase. However, at least two groups of investigators[18,108] observed that *A. hydrophila* counts increase with time even if the food is refrigerated (5° C). The presence of these bacteria in heated foods (pasteurized milk and ice cream, Table 38–5; ham, cooked tripe, and smoked cooked sausages, Table 38–8) likely reflects contamination of these foods after the heating step.

38.6 FACTORS AFFECTING SURVIVAL AND GROWTH, PARTICULARLY IN FOODS

38.6.1 Temperature

Most strains of *A. hydrophila* tested grew in culture broth over the temperature range of 4-5° C to 42° C.[109] *A. hydrophila* will grow in a variety of fresh foods stored at 5° C, including red meat and poultry, shell- and finfish, vegetables, and milk.[18,108,121] The bacterium not only grew at this temperature, but grew in the presence of the background flora of the food. Palumbo et al.[108] observed that *A. hydrophila* comprised 10% of the bacterial count of a poultry sample. The minimum growth temperature prob-

Table 38–4 Incidence of Members of the *A. hydrophila* Group in Various Shell- and Finfish

Source	*Organisms Isolated*	*Incidence, Number +/ Total Number (%)*	*Range (cfu g^{-1})*	*Reference*
Finfish (US)	*Ah**	10/11 (91%)	1.5 × 10^{2} to 1.6 × 10^{5}	108
Raw shucked oysters (US)	*Ah**	3/3 (100%)	9 × 10^{3} to 5 × 10^{5}	107
Carp (Japan)	*A. hydrophila, A. caviae, A. sobria*	13/20 (65%)	mostly below 10^{2}	152
Various seafoods and aquacultured products (Taiwan)	*A. hydrophila, A. caviae, A. sobria*	105/1088 (9.6%)	not available	151
River fish (India)	*Ah**	(43%)	not available	114

*Ah** indicates biochemical testing not done to speciate isolates beyond the *A. hydrophila* group.

Table 38–5 Incidence of Members of the *A. hydrophila* Group in Milk and Dairy Products

Source	*Bacteria Identified*	*Incidence, Number +/ Total Number (%)*	*Level*	*Reference*
Raw milk (Australia)	*A. hydrophila, A. caviae, A. sobria*	43/72 (60%)	not available	61
Pasteurized milk (Australia)	*A. hydrophila A. caviae, A. sobria*	7/183 (3.8%)	not available	61
Pasteurized milk (Brazil)	*A. hydrophila, A. caviae, A. schuberti*	10/35 (29%)	not available	36
White cheese (Brazil)	*A. hydrophila, A. caviae, A. sobria*	8/25 (32%)	not available	36
Ice cream (UK)	*A. caviae*	3/64 (4.7%)	not available	51
Whipped cream (Denmark)	*A. hydrophila, A. caviae, A. sobria*	9/32 (28%)	maximum level $>10^5$ cfu g^{-1}	65

Table 38–6 Incidence of Members of the *A. hydrophila* Group in Poultry

Source	*Bacteria Identified*	*Incidence, Number +/ Total Number (%)*	*Level*	*Reference*
Raw chicken (Japan)	*A. hydrophila, A. caviae, A. sobria, A. jandaei*	30/94 (32%)	generally below 10^2 cfu g^{-1}	152
Broiler carcasses (US)	*Ah**	(100%)	2.8×10^3 cfu/carcass to 5.8×10^4 cfu/carcass	11
Turkey (New Zealand)	*A. hydrophila*	5/6 (83%)	not available	50
Chicken (US)	*Ah**	6/8 (75%)	1.7×10^3 to 1.1×10^4 cfu g^{-1}	108
Ground chicken and turkey (Canada)	*A. hydrophila, A. caviae, A. sobria*	8/8 (100%)	3×10^{2} to 1.7×10^4 cfu g^{-1}	137

*Ah** indicates biochemical testing not done to speciate isolates beyond the *A. hydrophila* group.

Table 38–7 Incidence of Members of the *A. hydrophila* Group in Red Meat

Source	*Bacteria Identified*	*Incidence, Number +/ Total Number (%)*	*Level*	*Reference*
Minced meat (Switzerland)	*A. hydrophila, A. sobria, A. caviae*	32/34 (94%)	Maximum number, 3×10^4 cfu g^{-1}	39
Ground meats (US)	*Ah**	8/8 (100%)	6×10^3 to 8.2×10^4 cfuU g^{-1}	108
Ground meats (Canada)	*A. hydrophila, A. sobria, A. caviae*	19/23 (83%)	1.1×10^1 to 1.9×10^3 cfu g^{-1}	137
Lamb—various cuts (Australia)	*A. hydrophila, A. sobria, A. caviae, Aeromonas* spp.	46/54 (85%)	26% by direct plating; 59% by enrichment	81
Minced meat (Denmark)	*Aeromonas* spp.	10/40 (25%)	maximum level $>10^5$ cfu g^{-1}	65
Raw pork (Japan)	*A. hydrophila, A. sobria, A. caviae, A. jandaei, Aeromonas* spp.	42/100 (42%)	mostly $<10^2$ cfu g^{-1}	152

*Ah** indicates biochemical testing not done to speciate isolates beyond the *A. hydrophila* group.

Table 38–8 Incidence of Members of the *A. hydrophila* Group in Miscellaneous Foods

Source	*Bacteria Identified*	*Incidence, Number +/ Total Number (%)*	*Level*	*Reference*
Fresh vegetables (US)	*A. hydrophila, A. caviae*	8/21 (38%)	1×10^2 to 2.3×10^4 cfu g^{-1}	18
Meat delicatessen (Denmark)	*Aeromonas* spp.	12/236 (5%)	maximum level, 2×10^4 cfu g^{-1}	65
Cream cakes and desserts (Denmark)	*Aeromonas* spp.	10/177 (6%)	maximum level, $> 10^5$ cfu g^{-1}	65
Mayonnaise-based salads (Denmark)	*Aeromonas* spp.	18/177 (10%)	maximum level, $> 10^5$ cfu g^{-1}	65
Cooked tripe (UK)	*A. hydrophila*	21/44 (48%)	10^2 to 10^7 cfu g^{-1}	52
Ham (Switzerland)	*A. hydrophila, A. caviae, A. sobria*	13/34 (38%)	1×10^2 cfu g^{-1}	39
Smoked cooked sausage (Switzerland)	*A. hydrophila, A. caviae*	5/32 (16%)	$<10^2$ cfu g^{-1}	39

ably approaches the freezing point of individual foods; growth occurred in culture at 1° C[33] and at 0° C in purge (drip) from a lamb carcass.[41]

According to Popoff,[116] the optimum temperature for growth of the mesophilic aeromonads is 28° C. Palumbo[102] observed the same lag time at 28° C and 37° C (Table 38–9), with a shortest generation time at 28° C for one strain. A comparison of lag and generation times for 24 clinical and environmental isolates at 29° or 37° C showed that many strains had shorter lag times at one temperature and shorter generation times at the other.[140] The maximum growth temperature for most strains of *A. hydrophila* appears to be at least 42° C,[109] with most enterotoxigenic strains capable of growth at 43° C.[62]

38.6.2 Salt and Water Activity

Palumbo et al.[109] conducted one of the first comprehensive studies of the effect of salt on the growth of *A. hydrophila*. The highest level of NaCl that would allow growth was a function of pH and temperature. At 28° C, many *A. hydrophila* strains grew in the presence of 4.5% NaCl and pH 7.3; as the temperature decreased, the growth-limiting NaCl level also decreased. NaCl restricts growth of microorganisms by lowering the water activity (see Chapter 7). The minimum a_w that supported growth of three strains of *A. hydrophila* varied with temperature and humectant.[126] Growth was limited at higher a_w when NaCl was used than when polyethylene glycol was used to lower the a_w; the effect of glycerol was intermediate.

Table 38–9 Effect of Temperature on the Growth of *A. hydrophila* K144 in BHI Broth[102]*

Temperature ° C	*Generation Time (h)***	*Lag Time (h)***
42	1.21 ± 0.21	32.44 ± 5.57
37	0.53 ± 0.01	2.54 ± 0.76
28	0.40 ± 0.01	2.56 ± 0.15
19	0.86 ± 0.03	7.06 ± 0.46
12	1.52 ± 0.06	15.15 ± 2.21
5	3.62 ± 0.08	43.84 ± 0.85

*The inoculum was grown with shaking (200 rpm) overnight to early stationary phase (18 hr) in BHI broth at 28°C, diluted in 0.1% peptone broth, and inoculated into 50 ml quantities of BHI broth in 250 ml flasks to yield a starting count of *ca* 10^3 CFU ml^{-1}. Triplicate flasks were placed at each of the temperatures, shaken at 200 rpm, and sampled at appropriate intervals. Viable cell counts were performed by surface plating (with a Spiral Plater) onto duplicate tryptic soy agar plates, and colonies were counted after 24–36 hrs at 28° C. Growth kinetics were calculated using the Gompertz equation in conjunction with Abacus, a nonlinear iterative regression program.

**Average of triplicate flasks ± standard deviation.

Source: Copyright © 1997, S.A. Palumbo.

38.6.3 pH and Acidulant

The acid tolerance of strains is a function of both temperature and NaCl content of the growth medium.[109] At 28° C, 3 of 10 strains grew at pH 4.5 in medium acidified with HCl (within 3 days), while at 5° C, only 4 of 10 strains grew at pH 5.5 (within 13 to 17 days), and none grew at pH 4.5. The limiting pH of *A. hydrophila* was a function of different acidulants, with HCl being the most permissive.[110] The inhibitory activity of acids appeared to be related to their pK_as and followed the order from least inhibitory to most: HCl<H_2SO_4<tartaric<citric<lactic<acetic. Despite the apparent sensitivity of *A. hydrophila* to acetic acid in a culture system, the bacterium has been isolated from salads prepared with mayonnaise;[65] the pH of these foods averaged 5.8, which is borderline inhibitory for *A. hydrophila*[110] depending on salt level and storage temperature. It is not known whether the bacterium was present as a contaminant or some increase in numbers had occurred. When inoculated into pasteurized milk used for the manufacture of yogurt, *A. hydrophila* died off during storage of the product held at 4° C after fermentation.[9,10]

38.6.4 Nitrite

Inhibition of bacterial growth by nitrite is pH-dependent, with little or no inhibition at pH 6.0 or above, and, in general, nitrite is a much more effective inhibitor under anaerobic conditions.[20] The responses of *A. hydrophila* to nitrite are similar to those of other bacteria: maximum inhibitory activity is at pH 6.0 and below and maximum activity occurs under anaerobic conditions.[112,113] The isolation of the *A. hydrophila* group from cooked, sliced ham and mettwurst in product surveys[39] undoubtedly reflects post-processing contamination rather than growth in the product. The residual nitrite in most cured meat products is extremely low and probably has little inhibitory effect.

38.6.5 Gaseous Atmosphere

The *A. hydrophila* group is facultatively anaerobic.[116] These bacteria have been isolated from various anaerobically (vacuum) and modified atmosphere-packaged refrigerated fresh meats.[14,34,38,74,90,132,136] The background flora that develops during storage often has a greater controlling influence than atmosphere or even temperature.

38.6.6 Chemical Preservatives

Various compounds added directly or indirectly to foods can inhibit the growth of *A. hydrophila*. These include essential oils, liquid smoke, food preservatives, and polyphosphates. *A. hydrophila* was inhibited by the essential oils of clove, coriander, nutmeg, and pepper tested in both a model system and a noncured cooked pork.[141] The bacterium was inhibited by liquid smoke isolated from several species of wood.[139] *A. hydrophila* was the most sensitive of four psychrotrophic foodborne bacteria studied to the food preservatives methyl p-hydroxybenzoate and potassium sorbate.[87] *A. hydrophila* was sensitive to the food preservatives butylated hydroxyanisole, propylhydroxy parabenzoate, and sodium tripolyphosphate; protease secretion was more sensitive than growth.[148] Palumbo et al.[105] studied the effect of food polyphosphates (Sodaphos, Hexaphos, sodium pyrophosphate, and sodium tripolyphosphate) on the growth of the bacterium in both a model system and ground pork. In a culture broth, the polyphosphates caused small changes in lag and generation times; however, when polyphosphates were combined with 3.5% NaCl, the number of viable *A. hydrophila* declined. In contrast, most of the other compounds mentioned above simply inhibit the growth of *A. hydrophila* but may be useful when combined with other factors such as increased NaCl or lowered pH. Additional studies are needed to increase the effectiveness of compounds already used in foods.

38.6.7 Combination of Preservative Factors

Predictive modeling studies (see Chapter 16) have been made of the effect of combinations of temperature, pH, NaCl, nitrite, and atmosphere on *A. hydrophila*.[112,113]

38.6.8 Bacteriocins

Several lactic acid bacteria (LAB) cultures showed activity against *A. hydrophila* in a culture broth.[75] The bacteriocin(s) of a lactic starter inhibited *A. hydrophila* in both skim and ewe's milk.[127] Neither nisin (4000 activity units ml^{-1}) nor pediocin (4000 activity units ml^{-1}) alone was effective against *A. hydrophila*, but when combined with a preliminary heating or freezing treatment, the bacterium became sensitive.[57]

38.6.9 Heating

The heat resistance of *A. hydrophila*, as of other bacteria, is influenced by factors such as growth temperature, age of the culture, and heating menstruum (food or buffer).[29,111] The relatively few studies on the resistance/sensitivity of *A. hydrophila* to heat suggest that treatments such as milk pasteurization and frankfurter processing (heating to a final processing temperature of 160° F) should inactivate levels encountered in most raw foods. *A. hydrophila* was more heat sensitive than *Salmonella typhimurium* and *Escherichia coli* O157:H7 when heated in either hamburger or egg yolk.[95] Condon et al.[29] determined a $D_{55^\circ C} = 0.17$ min and a $z = 5.11^\circ$ C for single strain heated in buffer. Palumbo et al.[112] studied five strains (three clinical and two food isolates) heated in saline and raw milk and observed $D_{48^\circ C}$ of 5.2 and 4.3 min respectively and a $z = 6.21^\circ$ C.

38.6.10 Irradiation

Radiation *D*-values at 2° C of five strains of the bacterium in ground beef and blue fish ranged from 0.14 to 0.19 kGy.[107] This is similar to most other Gram-negative bacteria associated with food (see Chapter 4). Thus, any irradiation treatment given to a food to eliminate other Gram-negative pathogens should also eliminate *A. hydrophila*.

38.6.11 Chlorine and Disinfectants

Chlorine represents one of the major food processing plant disinfectants and the major, if not sole, disinfectant used in the final stage of water treatment in the US. Overall, *A. hydrophila* is more susceptible to chlorination than *E. coli*;[64] however, *A. hydrophila* can be isolated from chlorinated drinking water[16] as well as from water with a negative coliform test. Though there are several potential explanations, high organic content of the water may react with the chlorine and thus result in inadequate chlorination and inadequate destruction of the bacterium.[130]

38.6.12 Lactoperoxidase (LP) System

The LP system is effective against both Gram-positive and Gram-negative bacteria. Recent work by Santos et al.[125,127] indicated the potential of using the LP system to inactivate *A. hydrophila* (starting count 10^2-10^3 ml^{-1} or g^{-1}) in culture broth, skim and ewe's milk, and in a ewe's milk soft cheese. The LP system was effective also against the spoilage flora of these products, making it useful to extend product shelf life and protect against this pathogen.

38.7 PRINCIPLES OF DETECTION AND ISOLATION

38.7.1 General Considerations

Members of the *A. hydrophila* group can be isolated easily using most general, all-purpose bacteriological media and on various specific and differential media. On a nonselective medium such as tryptic soy agar, they are difficult to distinguish morphologically from members of the *Enterobacteriaceae*. Also, on differential media such as MacConkey agar, the *A. hydrophila* group resembles other non-aeromonads and may appear as lactose-positive or lactose-negative. The oxidase test is used generally to distinguish these bacteria from other closely related bacteria. The organism can be isolated readily from food and environmental and clinical specimens. Because mesophilic aeromonads can proliferate at refrigeration temperatures, immediate analysis of samples in the laboratory is preferred. If samples are to be analyzed within a day or two, they should be stored at refrigeration temperatures. Samples kept longer should be stored at –20° C or –72° C. Survival of mesophilic aeromonads at frozen temperatures has not been studied; however, Abeyta et al.[2] recovered *A. hydrophila* from oysters held frozen for 1.5 years at –72° C. An enrichment technique may be necessary when analyzing frozen foods, or foods that may contain injured bacteria, for the presence of mesophilic aeromonads.

38.7.2 Procedures and Sampling

Specific details on sample preparation and general isolation procedures are described elsewhere.[103]

An enrichment step is indicated if low numbers of mesophilic *A. hydrophila* group are anticipated or if the cells may have become injured, eg by heating or freezing. Two enrichment broths used successfully for the *A. hydrophila* group are alkaline peptone water (APW) and tryptic soy broth + ampicillin (TSBP). Although no studies comparing their effectiveness have been done, each appears adequate to recover the *A. hydrophila* group from foods. APW has been used for foods such as lamb,[81] ice cream,[51] and mixed seafoods;[151] while TSBP has been used for frozen oysters[2] and raw and pasteurized milk.[61] These four groups of investigators either did not determine the total bacterial count or it was low (1.1×10^3 cfu g^{-1}) so no comment can be made on how well these two enrichment broths perform in isolating the *A. hydrophila* group from foods with high total bacterial counts.

After enrichment and sample preparation, an aliquot of the food is plated onto a medium selective for the *A. hydrophila* group. The current plating medium of choice for food samples appears to be starch ampicillin agar (SAA).[108] SAA contains ampicillin at 10 µg ml^{-1} to suppress Gram-negative background organisms and starch as the differential substance (members of the *A. hydrophila* group are among the few amylase-positive bacteria in foods). SAA was developed in 1985 and has found widespread acceptance for the isolation of the *A. hydrophila* group from food. Various recovery media for the *A. hydrophila* group have been evaluated by comparing both quantitative recovery of these bacteria and ease of distinguishing *A. hydrophila* from other bacteria.[63,137,144] For example, 85% of presumptive *Aeromonas* colonies on SAA were confirmed, compared with much lower percentages on three other recovery media.[63] Singh[137] observed a higher recovery rate of the *A. hydrophila* group on SAA than on two other recovery media. Numerous workers have used SAA to recover these bacteria from milk, meats, vegetables, fish, and processed foods.[18,50,61,65,96,108,137]

However, some strains of the *A. hydrophila* group have been reported to be sensitive to ampicillin,[118] and other plating media may be needed to recover these bacteria. Several media have been used with clinical specimens.[149] Members of the *A. hydrophila* group are sensitive to bile salts, and thus any bile salts–containing media would yield a lower quantitative recovery.[15,108] Any prospective plating medium should be evaluated carefully to ensure both quantitative recovery of members of the *A. hydrophila* group and ease of distinguishing these bacteria from the background microflora.

Any isolate can be identified easily as belonging to the *A. hydrophila* group by using a simple single-tube medium[59] that combines seven biochemical reactions in the one tube. This medium offers good differentiation of *A. hydrophila* from the biochemically similar enterics. After performing the oxidase test, the following additional reactions will verify the identity of isolates: Gram-negative rods 0.3-1.0 µm × 1.0-3.5 µm, motile by a single polar flagellum, metabolism of glucose is both respiratory and fermentative (carbohydrates broken down to acid or acid and gas), and resistance to the vibriostatic agent at the 150 μg level.[116] See Table 38–1 for further distinguishing characteristics. Speciation of isolates is performed using appropriate biochemical tests (Table 38–2); this scheme allows identification of all currently known HGs. Because it is often not feasible to perform all the tests in Table 38–2, investigators have developed rapid identification schemes. These are based on a limited number of biochemical tests and appear particularly useful in the clinical laboratory.[1,19] A scheme based on 9 to 16 tests allowed 99% of the strains to be assigned to the correct HG.[1] Aerokey II, a simpler scheme of 7 tests (esculin hydrolysis, gas from glucose, acid from arabinose, indole production, acid from sucrose, Voges-Proskauer, and resistance to cephalothin [30 μg]), was proposed by Carnahan et al.[19] and permitted them to identify correctly 97% of the strains tested.

Rapid identification kits are available and have been compared for their ability to confirm *Aeromonas* isolates.[96] Of API 20E, API 20NE, and Microbact 24E, API 20NE and Microbact 24E correctly identified 96.3% of the food isolates studied.

38.8 CONTROL

Food microbiologists generally rely on the three "Ks" to control the incidence and level of undesirable microorganisms (pathogens and spoilage agents) in foods: keep them out of the food, keep them from growing in the food, and kill them. These principles are applied to control the incidence and level of the *A. hydrophila* group in foods. The widespread occurrence of the *A. hydrophila* group in various foods (Tables 38–4 to 38–8) at a high incidence although low levels suggests that it is difficult to keep them out of most foods. However, their low levels would suggest that food conditions (salt concentrations and pH and acid type combined with low holding temperatures) can restrict their increase. The source of these bacteria in foods may be contaminated water (Table 38–3) and/or animal feces.[42,144] However, because of their low incidence and level in animal feces,[42,144] water is probably the main source of these bacteria in foods.

The importance of water as a potential vehicle for these bacteria was suggested further by the demonstration that depending on holding temperature, organic content, and residual chlorine, *A. hydrophila* could remain viable for several weeks in various water supplies and even increase in number.[106]

It is difficult to prevent members of the *A. hydrophila* group from growing in most fresh foods, even when the foods are adequately refrigerated (5° C).[11,18,108] Although they have been detected in various cooked, cured, and fermented foods, their incidence and level tends to be low.[39,50,65] Some exceptions are known, however. For example, 10^7 CFU *A. hydrophila* g^{-1} were detected in cooked tripe, suggesting either gross contamination and/or gross temperature abuse of the product after cooking.[152]

Survey data[39,50,65,108] suggest that although members of the *A. hydrophila* group appear to be sensitive to the salt and pH (acid) content of foods, they will survive conditions found in fresh foods. Palumbo et al.[108] did not observe any increase in *A. hydrophila* in fresh pork sausage (salt level, *ca* 2%) over one week's storage at 5° C. In culture, *A. hydrophila* is sensitive to pH values of 5.5 and below, especially if the pH is adjusted with lactic or acetic acids.[110] The number of viable *A. hydrophila* decreased in ground pork when conditions favored growth and acid production by LAB.[100]

A. hydrophila has been detected in vacuum- and modified atmosphere–packaged meats. Because members of this group are facultative anaerobes, this is not unexpected. The fate of *A. hydrophila* in these meat products appears to depend on the competing microflora that develop. As indicated above, the numbers of viable *A. hydrophila* in ground pork decreased as developing LAB produced lactic acid.[99] Raw shucked oysters were one of the few fresh foods in which the numbers of *A. hydrophila* decreased during refrigerated storage. LAB developed during storage and converted carbohydrates present in the oysters to lactic acid and thus inactivated the *A. hydrophila* present.[108]

The presence of the *A. hydrophila* group in foods can be controlled by heating, irradiation, chlorine, disinfectants, and the LP system (see Sections 38.6.9 to 38.6.12) which inactivate or kill the bacterium. Control by pH and acidulant (See Section 38.6.3) is multifactorial, with factors such as NaCl level, temperature, and acidulant playing important roles. With regard to pH and acidulant, these bacteria are often inhibited rather than killed.

38.9 CONCLUDING REMARKS

The *A. hydrophila* group represents a challenge and enigma to both clinical and food microbiologists. *A. hydrophila* is a recognized cause of systemic infections in immunocompetent individuals and can cause specific problems in immunocompromised patients. The bacteria are recognized pathogens of fish. These bacteria occur widely in both aquatic and food environments where they often can reach fairly high numbers, even in foods properly refrigerated. Isolates form factors that are associated with virulence in other pathogens, and clinical isolates possess these same virulence-associated factors. Circumstantial evidence points to a role for these bacteria as agents of diarrheal disease even though conclusive epidemiological evidence is lacking. There is not a current animal model, and the one human volunteer feeding study gave inclusive results.

There are many areas where more information is needed to relate members of the *A. hydrophila* group and diarrheal disease. One suggested approach would include a detailed molecular epidemiological comparison of clinical and food and water isolates from the same geographic area and the same time period to determine the source of the patients' strains. A foodborne disease outbreak in which the isolate from food exactly matches the patient isolate would provide a further link between *A. hydrophila* group and diarrheal disease. Havelaar et al.[46] compared diarrheal and water isolates using three typing methods (biotyping, serotyping, and gas-liquid chromatography of cell wall fatty acid methyl esters). They found little overall similarity between diarrheal and water isolates and concluded that environmental (water) strains that can infect humans are rare and probably represent a special subset of those occurring in water.

Clinical isolates should be investigated for new and different virulence factors and mechanisms. Although research on the *A. hydrophila* group lacks the urgency of that on recognized pathogens, the potential exists for this group to become a major problem, considering its wide occurrence in the environment and its ability to grow at refrigeration temperatures. It is tempting to speculate that the *A. hydrophila* group is responsible for at least a portion of foodborne disease outbreaks of unknown etiology.

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39 *Bacillus* species

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39.10 Concluding remarks
References

39.1 INTRODUCTION

Bacillus cereus is a common soil saprophyte. It has been isolated from a wide variety of foods, especially of plant origin, but also from meat, fish, and dairy products.[42] It was first recognized as a foodborne pathogen in 1949, when Hauge[33,34] isolated this species from vanilla sauce following an outbreak of diarrheal food poisoning at a hospital in Oslo, Norway. The vanilla sauce was cooked the day before consumption and stored at room temperature until served. To demonstrate that *B. cereus* was the cause of illness, Hauge grew the isolated organism to a concentration of about 4×10^6 ml^{-1} and drank 200 ml of the cocktail. After 13 hours he developed abdominal pain and watery diarrhea; these symptoms persisted for about 8 hours.[34] More than 20 years later another form of *B. cereus* food poisoning was described by workers in England.[46] The symptoms were dominated by vomiting and a short incubation period (<5 h), indicating that this was an intoxication.[46] This disease is now referred to as *B. cereus* Emetic Syndrome.

During the last decade, psychrotrophic strains of *B. cereus* have been recognized that are able to grow at 4 to 6° C.[18,28,32,66,69] These organisms are potentially of concern in pasteurized milk and preprepared chill-stored meals. Also there have been a number of reports of food poisoning caused by four other *Bacillus* species: *B. thuringiensis, B. subtilis, B. licheniformis*, and *B. pumilis*.[41,42] Most strains of *B. thuringiensis* produce the same enterotoxins as *B. cereus*, but nothing has been reported concerning the virulence factors of the other species (Damgaard and Granum, unpublished).

39.2 TAXONOMY AND TYPING OF THE FOOD POISONING *BACILLUS* SPECIES

The genus *Bacillus* is taxonomically complex. The most studied *Bacillus* species belong to the mesophilic group with growth temperature optima between 30° and 45° C, while members of the thermophilic group may grow at temperatures as high as 65° C but often fail to grow below 45° C. All the food poisoning *Bacillus* spp. belong to the mesophilic group, which is further divided into three groups;[17,51] all the food poisoning strains belong to Group 1. This group is composed of Gram-positive species forming ellipsoidal or cylindrical spores, usually without distended sporangia; most species within this group will grow both aerobically and anaerobically. It is further divided into Group 1A and 1B

The authors wish to thank Dr R.J. Gilbert for providing additional material for this chapter.

based on vegetative cell dimensions and the presence of lipid globules in the cytoplasm. Group 1A consists of species with cell widths >0.9 μm, producing lipid globules and includes *B. cereus*, *B. megaterium*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis*. Group 1B contains species with cell widths <0.9 μm and without lipid globules, and includes *B. subtilis*, *B. licheniformis*, *B. pumilis*, and *B. coagulans*. This grouping of *Bacillus* spp. is taxonomically not satisfactory, and only DNA studies will reveal the true relationship of the species. Indeed, some studies already have suggested that *B. cereus*, *B. megaterium*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis* should be considered subspecies of the same species.[7,8,14,18] In the laboratory, however, it is useful to distinguish these species, which can be done using the characteristics listed in Table 39–1.

A serotyping scheme based on flagellar antigens has been developed for *B. cereus*;[62] 42 serotypes currently are recognized. Certain serotypes are more commonly involved in the emetic food poisoning–type (serotypes 1, 3, and 8) and others with the diarrheal type (serotypes 1, 2, 6, 8, 10, and 19).[22,42] Serotype 1 is most commonly involved in food poisoning worldwide but most serotypes have been implicated.[42] There are no known genetic relationships between *B. cereus* virulence factors and flagella antigens; such a relationship is unlikely. Consequently, serotyping is not particularly useful for identifying food poisoning strains. Phage typing,[4] plasmid analysis,[48] random amplified polymorphic DNA (RAPD)[59] and polymerase chain reaction (AP-PCR)[45] are useful for subtyping strains from food poisoning incidents.

39.3 CHARACTERISTICS OF HUMAN DISEASE

Bacillus cereus may cause many different infections and intoxications.[42] In addition to foodborne diseases, *B. cereus* causes septicemia, meningitis, and ocular infections.[19,42] The two types of food poisoning are caused by very different virulence factors. The emetic disease, characterized by a short incubation period (1 to 5 hours) is caused by a small cyclic polypeptide (cereulide)[3] preformed in certain foods (eg, rice). The symptoms include nausea, vomiting, and stomach cramps (Table 39–2). The diarrheal disease is characterized by an incubation period of 8 to 16 hours with symptoms including abdominal pain, watery diarrhea, and rectal tenesmus (Table 39–3). It is caused by the production of enterotoxin(s) during the vegetative growth of *B. cereus* in the small intestine following the ingestion of bacterial cells or spores.[24,29] Usually both types of food poisoning are relatively mild, lasting for less than 24 hours, and do not usually require medical attention. However, occasionally a more severe type of diarrhea may occur, which may be caused by colonization of the epithelial cells of the small intestine by *B. cereus* spores, followed by outgrowth and enterotoxin production.[5]

Food poisoning caused by *B. subtilis*, *B. licheniformis*, and *B. pumilis* is similar to that caused by *B. cereus* in duration and range of symptoms. However, the incubation period and symptoms vary between and within species (Table 39–7). *Bacillus thuringiensis* is believed to produce the same enterotoxin as *B. cereus*, but nothing is known about the food poisoning toxins of the other three *Bacillus* species.

39.4 EPIDEMIOLOGY

Since *B. cereus* food poisoning is not a reportable disease, the true incidence of illness is unknown. It was reported to be the cause of 33% of cases of food poisoning (excluding those caused by viruses) in Norway (1988–1993);[25] 47% in Iceland (1985–1992); 22% in Finland (1992); 8.5% in the Netherlands (1991); and 5% in Denmark (1990–1992).[55] Much lower figures have been reported from other countries including the United States (US) (1.3%) and Canada (2.2%).[42] In England and Wales, there were 63 outbreaks (468 cases) from 1990 to 1995; over the same period, there were 65 outbreaks and 4,474 cases in Japan (R. Gilbert personal communication, 1997). Many fewer cases of food poisoning involving other *Bacillus* spp. have been reported, but 180 incidents involving more than 900 cases were reported in the United Kingdom (UK) between 1974 and 1996

Table 39–1 Criteria To Differentiate between the Four Closely Related *Bacillus* Species

Species	*Colony Morphology Blood Agar*	*Hemolysis*	*Motility*	*Susceptible to Penicillin*	*Parasporal Crystal Inclusion*
B. cereus	White	+	+	–	–
B. anthracis	White	–	–	+	–
B. thuringiensis	White/gray	+	+	–	+
B. mycoides	Rhizoid	(+)	–	–	–

Table 39–2 Characteristics of the Emetic Disease and Properties of the Emetic Toxin from *Bacillus cereus*

Property/Activity	*Description*
Intoxication dose	Number of *B. cereus*: 10^5 to 10^8 cells per gram food Amount of toxin: 12–32 μg kg^{-1} (*Suncus murinus:* a small monkey)
Toxin produced	In foods (25 to 35° C) as a secondary metabolite (?)
Incubation period	½ to 5 h
Duration of the illness	6 to 24 h
Symptoms	Nausea, vomiting (sometimes followed by diarrhea, due to additional enterotoxin production?)
Most frequently implicated foods	Fried and cooked rice, pasta, and noodles
Name of toxin	Cereulide
Structure of toxin	Cyclic peptide: $[\text{D-O-Leu-D-Ala-L-O-Val-L-Val}]_3$
Molecular mass	1.2 kDa
Isoelectric point	Uncharged
Antigenicity	None
Biological activity on man	Emesis
Type of receptor	5-HT_3 (binding stimulates the vagus afferent nerve)
Result of ileal loop tests	None
Cytotoxic	No
Activity on HEp-2 cells	Vacuolation activity
Heat stability	90 min at 121° C (no loss of activity)
Effect of proteolysis	None
How is the toxin produced	Not known, but probably enzymatically

Source: Data from references 1, 3, 42, 58.

(Table 39–4); mainly *B. subtilis* and *B. licheniformis* are involved. The types of foods and illnesses involved in *B. cereus* food poisoning in different parts of the world reflect eating habits (Table 39–5). Thus, in rice-eating countries such as Japan, India, Korea, and Thailand, the emetic type of poisoning is most common. In Europe and North America, the diarrheal type is most common and involves vegetable and meat-based foods.[42] Although milk commonly is contaminated with *B. cereus* spores and toxin may form,[15] it is seldom involved in *B. cereus* food poisoning. The reason for this is believed to be that insufficient growth occurs before spoilage is apparent.[43]

39.5 MECHANISMS OF DISEASE

39.5.1 The Emetic Syndrome

The characteristics of disease and the properties of the emetic toxin are summarized in Table 39–2. The elucidation of the emetic toxin structure has led to new insights into this syndrome. The emetic toxin

Table 39–3 Characteristics of the Diarrheal Disease Caused by *Bacillus cereus*

	Property
Infective dose	Usual 10^5 g^{-1} or ml^{-1}
Toxin produced	In the small intestine of the host
Type of toxin	Protein
Incubation period	8 to 16 h (occasionally >24 h)
Duration of illness	12 to 24 h (occasionally several days)
Symptoms	Abdominal pain, watery diarrhea, sometimes with nausea
Most frequently implicated foods	Meat products, soups, vegetables, puddings/sauces, and milk/milk products

Source: Data from P.E. Granum, *Bacillus Cereus* and its Toxins, *Journal of Applied Bacteriology*, Symp. Supplement, Vol. 76, pp. 61S–66S, © 1994, Blackwell Science, Ltd., and J.M. Kramer and R.J. Gilbert, *Bacillus cereus* and Other *Bacillus* species, in *Foodborne Bacterial Pathogens*, M.R. Doyle, ed., pp. 21–70, © 1989, Marcel Dekker.

has been named cereulide and is a cyclic peptide of three repeats of four amino-and/or oxy-acids (Table 39–2). The ring structure (dodecadepsipeptide) has a molecular mass of 1.2 kDa and is chemically closely related to the potassium ionophore valinomycin.[1,3] The biosynthetic pathway of the emetic toxin has yet to be elucidated, but its mode of action seems to be through stimulation of the vagus afferent nerve by binding of the 5-HT$_3$ receptor.[3] It is not clear whether the toxin is a modified gene product or is produced enzymatically in the cell or through modification of components of the growth medium. However, with such a structure, it is most likely that cereulide is an enzymatically synthesized peptide and not a gene product.

Previously it was believed that the emetic toxin was a lipid structure[24,42,57] because of its hydrophobic nature. Because of the structure of the emetic toxin, it is not surprising that it is not antigenic and is resistant to heat, pH, and proteolysis (Table 39–2). It is possible that other emetic toxins may exist, and these may be closely related structures.

Table 39–4 *Bacillus* Species Associated with Food Poisoning in England and Wales, 1974–1996

Species Implicated	*Incidents*	*Cases*
Bacillus cereus	108	445
B. licheniformis	35	246
B. pumilis	13	45
B. sphaericus	8	88
B. brevis	3	6
Mixed *Bacillus* spp	13	163
Total	**180**	**993**

Courtesy of R. Gilbert.

39.5.2 The Diarrheal Syndrome

The diarrheal syndrome is caused by at least two enterotoxins produced during vegetative growth of *B. cereus* in the small intestine.[24,29] Formation of sufficient enterotoxin in the food to cause food poisoning is theoretically possible, but such food would usually not be acceptable for human consumption. For this to occur, the numbers of *B. cereus* present in the food must be at least 10^6 g^{-1} or ml^{-1},[16,42] and sufficiently high amounts of enterotoxin must be formed to survive the pH of the stomach and the proteolytic enzymes of the duodenum.[24,29] These factors will rapidly reduce the enterotoxin activity to less than 1% of the initial level.[24,29] The relatively long time between the ingestion of the organism and occurrence of disease symptoms also supports the view that *preformed* enterotoxin is unlikely to be a factor in this syndrome.

The characteristics of the diarrheal syndrome are summarized in Table 39–3. The variation in the infective dose may be due to the ability of different strains to produce enterotoxin(s) and to the different susceptibility of individuals.

In Norway, two outbreaks have occurred in which many people were affected after eating stew (meat, potatoes, and vegetables). The infective dose was approximately 10^4 to 10^5. In the first outbreak (1992), 17 of 24 people exposed were affected and three of the patients were hospitalized, one for three

Table 39–5 Examples of Outbreaks of *Bacillus cereus* Food Poisoning, 1991–1996

Year	*Country*	*No. Persons Affected*	*Food Vehicle*	*Type of syndrome*
1991	India	27	Cooked rice	Emetic
1991	India	78	Cooked rice	Emetic
1991	Japan	1,877	"School lunch"	Emetic
1991	US	139	BBQ pork	Diarrheal
1992	Norway	17	Meat, potato, veg. stew	Diarrheal
1992	Spain	13	Cooked noodles	Diarrheal
1992	UK	21	Rice salad	Emetic
1993	US	14	Chicken and fried rice	Emetic
1993	Japan	39	Boiled rice and vegetables	Emetic
1994	US	87	Cooked rice and macaroni	Emetic
1994	UK	24	Noodles and meat	Emetic
1994	UK	21	Cooked rice	Emetic
1995	Norway	152	Meat veg. stew	Diarrheal
1996	India	45	Cooked rice	Emetic

Courtesy of R. Gilbert.

weeks;[5,23] the onset of symptoms was late (>24 h) in these three patients. The second outbreak was in February 1995 when 152 of 252 people were affected during the Norwegian junior ski championships.[27] The young competitors (16 to 19 years of age) suffered severe symptoms, while their coaches and the officials were not affected at all. Some of the skiers became ill after an incubation period of more than 24 hours and were ill for from one to several days.

The *B. cereus* spores from the first outbreak described above are distinctive in that they have the ability to adhere to Caco-2 cells (human intestinal epithelial cells).[5] After adhesion, the spores quickly germinate (within 1 hour), forming a vegetative *B. cereus* cell monolayer on top of the epithelial cells, followed by *in situ* production of enterotoxin. If this occurred in the intestinal tract, the enterotoxin concentration localized in the periphery of the tract would be much higher than in the lumen and thus cause greater damage to the tract and greater severity of disease. One would expect that with such a mechanism, the incubation period would be longer, as was observed. The local defense mechanisms would then determine the severity of the disease. The three people hospitalized in the first outbreak were over 50 years old and, in the second, only the young athletes were ill. It is likely that athletes in intense training are under stress and are perhaps more susceptible to intestinal infections.

The number and types of enterotoxins involved in the diarrheal form of *B. cereus* food poisoning have been the subject of debate for many years and remain controversial.[10,12,24,42,50,63,64] Both single- and multicomponent enterotoxins have been suggested to be the cause of illness.[2,12,24,31,42,44,56,63] Two different three-component enterotoxins are produced by food poisoning strains of *B. cereus.*[12,35,44,63] Several groups also have also described single-component enterotoxins with molecular weights of about 40 to 100 kDa.[12,20,24,31,42,63,64] However, the activities of these latter proteins may be due to contamination with traces of other proteins. The current knowledge of *B. cereus* enterotoxins is summarized in Table 39–6.

The early studies on enterotoxins[42,64] suggested that either a single-component or multicomponent toxin was involved. Further studies supported the hypothesis of a multicomponent toxin,[63] and a three-component hemolysin with enterotoxin activity was purified and characterized.[9,10,12] Although good evidence was presented that all three components were necessary for maximal enterotoxin activity,[12] other workers[31,55] have continued to claim that a one-component enterotoxin is responsible for all the activity. Since similar purification procedures were used by these two groups, it is tempting to suggest that they purified the same protein. By sequencing (Table 39–6), it has been shown that the protein purified by Granum and Nissen[31] is identical to the B component (37.8 kDa protein) of the hemolysin HBL purified by previous workers.[9,10,12,35] Since the B component and the L_1 component (38.2 kDa protein) have similar molecular weights, it is quite possible that the protein (B component) purified by the two groups was contaminated with the L_1 component. It is, however, more difficult to understand a possible contamination with the L_2 component. Until all these proteins can be expressed separately (after cloning), it is difficult to reach any conclusions on

Table 39–6 Properties of Enterotoxins Isolated from *Bacillus cereus*

Enterotoxin/Reference	*Molecular Weight*	*N-Terminal Sequence*	*Biological Test Used*	*DNA Sequenced*	*Hemolytic*
One main protein and two other protein components[42,64]	50 kDa	ND[1]	Positive on: rabbit loop tests, vascular permeability tests, and mouse lethality tests	No	Yes
Three components[63]	38.0 kDa 39.5 kDa 43.0 kDa	ND ND ND	Positive on: rabbit loop tests, vascular permeability tests, monkey feeding tests, and Vero cell test	No	Yes
One component[56]	45 kDa	ND	Positive on: mouse loop tests, vascular permeability tests, and mouse lethality tests	No	No
One toxic component and one non-toxin component[31]	40 kDa 48 kDa	IEQTNNGDTALSANE[2] XTQQEGMDISSSLXK	Positive on: Vero cell test and Caco-2 (human intestinal epithelial cells) cell test	No	No
Three components[10,12,35]	43.2 kDa 38.5 kDa 37.8 kDa	ETQXENMDIXS XETIAQEQKVGNYALGPE SEIEQTNNGDTALSANEA	Positive on: rabbit loop tests, vascular permeability tests	Yes, the 37.8 kDa protein (B-component)	Yes
One component[2]	41 kDa	MKELVSTARIGRSLGIHL	Positive on: mouse loop tests, vascular permeability tests, and mouse lethality tests	Yes	?
One component[20]	100 kDa	ND	Positive on: vascular permeability tests Negative on: rabbit loop tests	No	?
Three components[44]	105 kDa 45 kDa 39 kDa	ND AQNVIAPNTLSNSIRMLG AESTVKQAPVXAVAKAYN	Positive on: Vero cell test and Caco-2 (human intestinal epithelial cells) cell test	No	No

[1] Not done.

[2] Amino acid sequence.

how many components must be present for biological activity. Given that only a very few molecules of an additional component can result in biological activity, nothing can be ruled out on the structure and cooperation of the components of this enterotoxin. However, further support for three component enterotoxins was recently provided by Lund and Granum,[44] who purified three other components as part of a nonhemolytic enterotoxin. Two of these proteins are close in molecular weight to that observed for the HBL enterotoxin (Table 39–6), but have different N-terminal sequences. The third protein is much larger (105 kDa) and may possibly be the same as the protein purified by Ezepchuk et al.[20] Interestingly, none of these three proteins cross-react with the L_2 component.

Some strains of *B. cereus* produce two enterotoxins, while other strains produce only one,[26] but we do not know the distribution of the two enterotoxin complexes among strains or how important each is in relation to food poisoning. It is possible that at least some of the components of the two enterotoxins may substitute for each other and still remain enterotoxic. There is some sequence similarity between the L_1 component and the 39 kDa protein purified by Lund and Granum.[44]

Enterotoxin T is a single-component enterotoxin that has never been shown to cause food poisoning.[2] The enterotoxin T gene recently was shown to be absent in 57 of 95 strains of *B. cereus* and in 5 of 7 strains involved in food poisoning.[26]

39.5.3 Other *Bacillus* Species

Bacillus cereus and *B. thuringiensis* food poisoning is caused by the same types of enterotoxin(s). Nothing is known of the virulence factors (toxins) involved in the three other *Bacillus* spp. that are reported to cause food poisoning; the little that is known about these types of food poisoning is summarized in Table 39–7. The incubation period and symptoms vary among and within species. This may be indicative of more than one virulence factor, as the described symptoms indicate both emetic and diarrheal toxins may be involved.

39.6 INCIDENCE IN THE ENVIRONMENT AND IN FOODS

Spores and vegetative cells of *B. cereus* and the other food poisoning *Bacillus* spp. occur widely in soils (soil has been shown to contain 10^5 to 10^6 spores g^{-1}) and may be found in virtually all raw and processed foods with the exception of those sterilized by heat or irradiation. In a comprehensive study of the occurrence of *B. cereus* in foodstuffs, Nygren[49] examined 3,888 food materials, finding contamination rates of 51.6% in food ingredients, 44% in milk and dairy products, and 52.1% in meat and vegetable products. Contamination levels vary widely ($<10^1$ g^{-1} to 10^7 g^{-1}) and are summarized in Table 39–8.

Bacillus cereus is a particular problem to the dairy industry. It contaminates the udders of cows while they graze in the fields, contaminating the milk during milking (see Chapter 22). Spores are more hydrophobic than spores of other *Bacillus* spp., which makes them adhere particularly well to many types of surfaces.[6,38,39] This makes them particularly difficult to remove during cleaning and a difficult target for disinfection. *B. cereus* spores also have appendages or pili that are, at least partly, involved in adhesion.[38,39] Not only can these properties allow the spore to survive sanitation but they can also allow them to adhere to the epithelial cells of the intestinal tract (see Section 39.5.2).

The use of spores of *B. thuringiensis* as an insecticide may cause some confusion as this organism is indistinguishable from *B. cereus* on most microbiological media. It is important that nonenterotoxin strains of *B. thuringiensis* are used for crop spraying as enterotoxin-producing strains have caused food poisoning.[19,41] Consignments of vegetables containing spores of enterotoxigenic *B. thuringiensis* have been returned from Scandinavia back to the producing country, where their use as an insecticide is allowed (Granum, unpublished).

39.7 GROWTH, SURVIVAL, AND DESTRUCTION IN FOODS

Most strains of *B. cereus* are mesophilic and are able to grow in low-acid foods at temperatures down to 15° C and up to 55° C (optimum 30° to 40° C).[15,28,32,40] Some are psychrotrophic and able to grow at temperatures down to 4° to 6° C (maximum 30° to 35° C).[66,69] Spores are variable in their resistance to heat. Most are moderately heat-resistant ($D_{121°C}$ of 0.03 minutes), but some are markedly heat-resistant ($D_{121°C}$ of 2.35 minutes).[40] Spores of sero-

Table 39–7 Characteristics of the Illness Caused by the Three *Bacillus* Species Other Than *B. cereus* Causing Food Poisoning

	Bacillus subtilis	*Bacillus licheniformis*	*Bacillus pumilus*
Foods involved	• Meat dishes with elements of vegetables • Seafood and rice • Bread and pastry products • Sandwiches • Pizza	• Meat dishes with elements of vegetables • Bread and pastry products • Chicken	• Meat products • Sandwiches • Canned tomato juice
Infective dose	$>10^5$	$>10^6$	$>10^6$
Incubation period	10 min to 14 h	2 to 14 h	15 min to 11 h
Duration	2 to 8 h	6 to 24 h	
Symptoms			
• Vomiting	80%	54%	Yes
• Diarrhea	49%	92%	Yes (not all)
• Abdominal pain/cramps	27%	46%	?
• Other symptoms	Nausea, headaches, and flushing/ sweating	Not reported	Not reported

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Table 39–8 Incidence of *Bacillus cereus* in Raw and Processed Foods

	Range g^{-1} or ml^{-1}
Rice and Rice Dishes	
Raw rice	10^2–10^3
Boiled rice	10^1–10^7
Fried rice	10^1–10^5
Milk and Dairy Products	
Raw milk	10^1–10^2
Pasteurized milk	10^1–10^3
Milk powder	10^2–10^3
Cheddar cheese	10^1–10^2
Ice cream	10^1–10^3
Vegetable Products	
Spices and herbs	10^1–10^5
Pulses and cereals	10^2–10^5
Salad vegetables	10^1–10^3
Meat and Meat Products	
Raw meats	10^1–10^2
Hamburger	10^1–10^3

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type 1 tend to be more heat-resistant than other serotypes.[49] In oil, $D_{121°c}$ values of up to 30 minutes may be obtained for spores. The emetic toxin of *B. cereus* is markedly resistant to heat and has been reported to survive 126° C for 90 minutes;[65] in contrast the diarrheal toxin is heat-sensitive and inactivated by heating at 56° C for 5 minutes. The diarrheal toxin may be produced by psychrotrophic strains at temperatures down to 4° C.[69]

Growth may occur between pH 5.0 and 8.8 (optimum 6.0 to 7.0) and at a_ws down to 0.93 depending on the acidulant and humectant.[40] Predictive models have been published showing the interactive effects of temperature, pH, sodium chloride, and carbon dioxide on the growth of *B. cereus*.[60] Spores are moderately resistant to disinfection (eg, *D*-value of 0.4 minutes in the presence of 150 mg kg^{-1} available chlorine at pH 7.0).[40]

Other food poisoning *Bacillus* spp. have similar growth and resistance characteristics.

39.8 DETECTION AND ENUMERATION

39.8.1 Enumeration of the Organism

Most isolations of *B. cereus* from foodstuffs and environmental samples are made by direct plating onto selective and diagnostic media. There are many such media,[68] but those most widely used are Mossel's mannitol-egg yolk-polymyxin agar (MYEPA)[47] and Holbrook and Anderson's polymyxin-pyruvate-egg yolk-mannitol-bromothymol blue agar (PEMBA).[36] Both media rely on the hydrolysis of egg yolk lecithin and absence of acid production from mannitol as diagnostic features for *B. cereus* and the use of polymyxin B (100 IU ml^{-1}) as the selective agent. The replacement of the phenol red in MYEPA by bromothymol blue in PEMBA gave better differentiation of *B. cereus* than other *Bacillus* spp., this differentiation being further improved by reducing the peptone concentration to 0.1%; actidione (40 µg ml^{-1}) is used in media when moldy food is examined. False negative reactions (lecithinase-negative strains of *B. cereus*) may occur, and overgrowth by acid-producing microorganisms may make the identification of *B. cereus* colonies difficult. False positives may occur, so confirmation tests should always be carried out. These may be rapid biochemical tests using the range of tests shown in Table 39–9 or the staining of cells from a suspect colony with a lipid stain followed by microscopic examination of the cells for the presence of lipid globules.[36] Replacement of the bromothymol blue in PEMBA by bromocresol purple, and reducing the incubation time from 48 to 24 h, has been claimed to improve the isolation of *B. cereus* from some foods.[61]

39.8.2 Detection of the Food Poisoning Toxins

The emetic toxin only can be detected by observing symptoms when food extracts are fed to primates or by treating HEp-2 cells with extracts and examining for vacuolation of the cells.[37]

Two commercial kits are available for detecting the diarrheal toxin. One, the Oxoid *Bacillus cereus* RPLA enterotoxin detection kit (Oxoid Ltd, Basingstoke, England) and, the other, the Tecra bacillus diarrheal enterotoxin visual immunoassay kit (Bioenterprises Pty, Australia). The Oxoid kit detects the L_2-components of one of the two, three-component enterotoxins that have been characterized (Beechar and Wong[10] in Table 39–6), and the Tecra kit detects the 45-kDa component of the other three-component enterotoxin (Lund and Granum[44] in Table 39–6). Thus neither test specifically detects the biologically active toxin[11,44] although the results of the Tecra kit correlate with cytotoxicity.[16]

39.9 PREVENTION AND CONTROL OF *BACILLUS* FOOD POISONING

Bacillus cereus food poisoning is principally associated with the storage of cooked foods at tempera-

Table 39–9 Identification of Common Food Poisoning Strains of *Bacillus* species[53]

	B. cereus	*B. pumilis*	*B. subtilis*	*B. licheniformis*
Anaerobic growth	+	–	–	+
Mannitol[1]	–	+	+	+
Arabinose	–	+	+	+
Xylose	–	+	+	+
Nitrate reduction	+	–	+	+

[1] Acid production in minerals salt-based medium

Source: Reprinted with permission from D. Roberts, W. Hooper, and M. Grenwood, *Practical Food Microbiology*, © 1995, Public Health Laboratory Service.

tures and for times that allow growth to occur and the number of vegetative cells produced to exceed 10^6 ml^{-1} or g^{-1}. Thus rapid cooling of cooked foods to <10° C is generally an effective control measure. Low pH foods (< pH 5.0) and dry foods will not support the growth of *B. cereus,* although many dried foods will be contaminated with spores of this microorganism.

There are many reports of food poisoning occurring when dried foods are rehydrated and stored at temperatures at which growth can occur. The emetic syndrome form of *B. cereus* food poisoning, often associated with consumption of rice in Chinese restaurants, is an example of food poisoning arising from such a practice. A common cause results from saving portions of boiled rice from one day to the next and then using it to produce fried rice. The rice often is stored at room temperature as this results in the best form of rice for frying, but also provides ideal conditions for the growth of *B. cereus.* Educating Chinese restaurant owners to cool boiled rice in thin layers after bulk cooking and then refrigerating any rice stored from one day to the next has significantly reduced the incidence of food poisoning associated with the consumption of rice in Chinese restaurants in the UK.

The frequent contamination of milk with spores of psychrotrophic strains of *B. cereus* is of concern to the dairy industry as the growth of such spores causes spoilage (aggregation of the fat in the cream layer of pasteurized milk, often called "Bitty Cream") and "Sweet Curdling" (without pH reduction) of homogenized and nonhomogenized milk). *B. cereus* contamination of pasteurized milk is also potentially a food poisoning concern. However, evidence from trials of human volunteers consuming milk naturally contaminated with *B. cereus* and stored at refrigerated temperature indicates that the risk of *B. cereus* food poisoning from chill-stored refrigerated milk is low.[43] However, it is important to keep the level of *B. cereus* in milk as low as possible by cleaning the udder and teats of the cows prior to milking; cleaning, and disinfecting milking equipment, pipework, and storage vessels and by the storage of the milk at temperatures and for times that prevent *B. cereus* concentrations exceeding 10^4 ml^{-1} (see Chapter 22). Similar considerations to the control of *B. cereus* in milk apply to the control of *B. cereus* growth in preprepared, extended shelf life, chill-stored meals.

Food poisoning caused by the *Bacillus subtilis* group of organisms also is associated with poorly stored cooked foods. Vehicles include meat pies, rice dishes, and other cereal-based products such as bread and pizzas (Table 39–7). Control measures are the same as those applied to *B. cereus.*

39.10 CONCLUDING REMARKS

Bacillus cereus food poisoning is widespread, with the diarrheal type found mainly in the Western hemisphere and the emetic type in the Eastern hemisphere; this difference reflects the different types of foods consumed in these countries. The symptoms of poisoning are typical of many gastrointestinal infections and intoxications, these being relatively mild and of short duration. Principal foods of concern are dairy and cereal-based products. The psychrotrophic strains capable of growth at refrigerated food temperatures are of concern in all low-acid, extended shelf life, chill-stored foods. Further research is needed into the ability of these organisms to grow in these foods and to produce food poisoning toxins. The types, structure, and composition of the enterotoxins of *B. cereus* need much further study. There is a need for an *in vitro* test for the emetic toxin (cereulide) and a specific test for biologically active diarrheal toxin(s).

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40

Campylobacter

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40.1 INTRODUCTION

The disease-causing, enteropathogenic *Campylobacter* spp., transmitted primarily through foods to humans, consist mainly of the species *C. jejuni*, but may also include *C. coli, C. lari*, and *C. upsaliensis*.[83] We shall refer to these four species as campylobacters in this chapter. These fascinating, and all too frequently occurring human pathogens, have previously been labeled as "newly emerging" or "newly recognized." In 1957, E.O. King was first to suggest that human infections were caused by what she called "related vibrios." Twenty-five years after being emergent and becoming substantially recognized[28] as the most frequently isolated bacterial agent implicated in human gastroenteritis, such a label no longer fits.

Campylobacters are easily destroyed by drying,[51] relatively modest cooking, or processing procedures.[83] The organisms are fragile and pose a health problem only if foods are prepared using poor hygiene or are consumed before being thoroughly cooked. Consequently, the food handler should be able to prevent transmission through food products.[38]

The primary ecologic niche for campylobacters is the intestinal tract of warm-blooded animals. Their minimum growth temperature is about 30° C;[93] therefore, there are few situations in which they are likely to multiply in foods. Measures to reduce contamination with campylobacters during animal production, and during the processing of raw products, should be used as the main means of reducing the incidence of illness caused by these organisms.

40.2 TAXONOMY AND CHARACTERISTICS OF THE ORGANISMS

Campylobacter characteristically is Gram-negative, has narrow (0.2 to 0.5 µm) and long (1.5 to 5.0 µm) rods, is spiral-shaped and nonspore-forming, and contains a single polar flagellum on one or both ends that produces the characteristic corkscrew-like motility. *Campylobacter jejuni, C. coli*, and *C. lari* cause more than 90% of the reported human disease. These species grow well at temperatures ranging from 37° C to 42° C, but should not be considered as thermophilic because they die at temperatures at or above 45° C. Older cultures often become coccoidal. It is unclear whether this morphological change confers an advantage toward survival or whether this is cellular deterioration leading eventually to death. Viable, nonculturable forms may arise under conditions of starvation.[149] The genus is oxidase-positive and reduces nitrate to nitrite. The organism does not ferment or oxidize carbohydrates; it derives its energy through accessing a variety of tricarboxylic acid (TCA) intermediates or by decarboxylation and deamination of amino acids. Although oxygen is characteristically the terminal electron acceptor in the TCA cycle, the organism does not tolerate levels of oxygen normally found in the atmosphere, but grows optimally in microaerobic conditions. Most enteropathogenic campylobacters are catalase-positive. The organisms are thus ideally suited to their primary niches in the reproductive and intestinal tracts of warm-blooded animals.[126]

40.3 CHARACTERISTICS OF THE DISEASE

Symptoms of infection in humans are usually severe: watery and/or bloody diarrhea; abdominal pain that can be of such severity as to mimic appendicitis; and malaise, fever, and headaches also occur. The incubation period following ingestion is 2 to 5 days. Diarrhea caused by campylobacteriosis can vary from merely loose to profuse stools, which may contain blood, be slimy, and may be extremely foul smelling. Individuals often experience more than eight bowel movements in a day. Symptoms usually subside in less than 7 days, and are most severe 2 to 5 days after onset. Active excretion of the organism and disease symptoms can recur.[20,29] An infectious dose for humans may be as few as 500 cells.[11,146] However, considerable variation exists because of differences in virulence of campylobacter strains and in host resistance. Campylobacteriosis is typically a self-limiting disease. Antibiotic therapy should be considered only if complications and recurring episodes occur; erythromycin is frequently a highly effective treatment.[20] Hospitalization due to campylobacteriosis is reported less often than for salmonellosis or listeriosis but may account for 10% of cases; deaths are rare.[36]

40.4 MECHANISM OF PATHOGENICITY

The mechanism of pathogenicity is unclear. Because clinical symptoms of *Campylobacter* infections resemble those manifested by other enteropathogens, it is reasonable to presume that similar mechanisms may be involved. *Campylobacter* infection of humans results in high titers of humoral IgG, IgM, and IgA.[92] This could indicate that bacterial cellular constituents pass from the intestinal tract across the epithelium. Once on the serosal side, these antigens are presented to the immune system receptors and the cellular and humoral constituents are able to respond. Poor antibody response to *Campylobacter* infection is associated with patients who have prolonged or intermittent enteritis.[153]

Campylobacter diarrhea in developed countries is characterized by polymorphonuclear leukocytes in 78 to 93% of cases and blood in diarrhea specimens from 60% to 65% of the cases.[14] This is very different from the watery, noninflammatory diarrhea frequently observed in tropical regions.[32,64,176] The mechanisms of pathogenesis of *Campylobacter* may include association with and attachment to the intestinal epithelium, invasion of the epithelium, and/or toxin formation. Guerrant et al.[69] studied 12 isolates of *C. jejuni* from individuals with inflammatory diarrhea, all of which produced factors lethal to HeLa cells; four were cytotoxic also to CHO cells.[98] Cytotoxicity was not neutralized by the Shiga toxin antiserum. The mechanism and the receptor involved in this cytotoxicity are not yet known.

Enteropathogenic *Campylobacter* adhere to epithelial cells.[83,155] Adherence is related to an outer membrane protein (OMP) with a molecular weight of 27 kDa that binds to the host membrane. The outer membrane protein may be a major virulence factor. Other potential virulence factors have also been reported.[111] Ruiz-Palacios et al.[153] reported a strong association between enterotoxin production by *Campylobacter* isolates and illness; however, they did not find an association between cytotoxin production and diarrhea. They suggested that the mechanisms of interaction and the membrane receptors may be different for adherent and invasive strains. There is evidence that isolates from developing and developed countries show similar enterotoxicity, and it was suggested that the difference in clinical symptoms could be explained by the immune status of the exposed individuals affected.[138]

Campylobacters reside in the deep crypts lining the intestinal tract. The organism is highly motile and its spiral shape allows movement into the crypts of Lieberkuhn through the mucin layer adjacent to the gut surface.[9,104] Hugdahl et al.[78] demonstrated components of gastric mucin (especially L-fucose and L-serine) to be chemotactic attractants; Nachamkin et al.[120] reported that campylobacter flagella served a critical role in the colonization of chicks. Mutants without flagella were unable to colonize chicks, while the wild type readily did so. Although pathology is not manifested in chickens, the first phases in mucosal colonization may be similar in poultry and in humans. Consistent, inexpensive, and readily available animal virulence models are lacking and, as indicated above, various tissue culture assays showing enterotoxin or cytotoxin activities of the organism have been inconsistent and have yielded controversy.

Increasingly reported are the far more serious sequellae to the initial acute phase of enteritis. *Campylobacter* infections can cause reactive arthropathies, Reiter's syndrome, Guillain-Barré syndrome (GBS), and associated neurological disorders. People infected with the human immunodeficiency virus are particularly susceptible to *Campylobacter* enteritis[163] just as they are to other infections. In Los Angeles County, the incidence of *Campylobacter* enteritis in acquired immune deficiency syndrome patients was 519 patients per 100,000, whereas in general population it was 13 per 100,000 individuals.

Infection with *C. jejuni* can also lead to reactive arthritis.[35,154,185,187] This involves the inflammation of joints at a location distant from the enteric site of infection. The inflammation is sterile and develops within days to weeks after the acute phase of infection. Antibody levels against *C. jejuni* are elevated but the organism cannot be isolated from the infected joint.

The costs of *Campylobacter* enteritis each year within the United States (US) are estimated from $156 million[181] to $4 billion.[60] In 1995 the annual costs of campylobacteriosis in the US were estimated to range from $362 to $699 million.[27] More recently, Buzby and colleagues estimated costs for cases of US campylobacteriosis as ranging from $1.17 billion to $1.37 billion annually.[31] A study in 1997 estimated that reported incidents incurred costs of at least 4.5 million dollars and that actual figures may be 10 times this amount for New Zealand. These estimates do not include the enormous expenses associated with the sequellae attributed to *Campylobacter* infection. Nor do they include the pain and suffering endured by both the infected individual and their families who are afflicted with these complications of the enteric infection, such as motor axonal neuropathy, Reiter's syndrome, and GBS.[58,114]

40.5 INCIDENCE OF THE ORGANISMS IN THE ENVIRONMENT AND IN FOODS

40.5.1 General Incidence in the Environment

Animals used for food production and domestic pets often are reservoirs for campylobacters.[21] These bacteria generally are commensal organisms in the intestinal tract of a wide variety of animals; however, they occasionally serve as enteric pathogens in the young of some species such as calves, lambs, and puppies.[159] *Campylobacter jejuni* is found commonly in the intestinal tract of birds, cattle, and sheep, whereas *C. coli* is most often associated with pigs.[46] In developed countries, asymptomatic excretion of campylobacters by human carriers is uncommon; therefore, humans serve as only a minor reservoir. However, humans may transfer campylobacters into animal production and food-processing areas on contaminated clothing (boots or gloves) or through inadequate personal hygiene. In developing countries, where proper hygiene is often more difficult to practice, human carriage may be more important in disease transmission.[21] A study in Washington state showed that campylobacters may be isolated from environmental sources such as ponds, lakes, and creeks.[33] No correlation was found between incidence of campylobacters and fecal bacteria. Generally, higher rates of isolation were noted in fall and winter when the water temperature is lower, extending the time the organism can survive outside the intestinal tract of its animal host.[33] Unhygienic water sources have been associated with numerous outbreaks of campylobacteriosis where adequate levels of chlorination and the operational integrity of community water systems were not maintained properly.[112,148,155,180] Campylobacters have been isolated from sea water, streams, rivers, and estuaries that have been tainted by fecal contamination from wild or domestic animals.[98] Campylobacters survive better in cool, fresh water[17] than in warm or brackish water.[98] Harris[73] determined that raw milk and mushrooms were the only nonmeat foods consumed that were linked to campylobacteriosis. Doyle and Schoeni[52] isolated *C. jejuni* from only 3 of 200 (1.5%) packages of retail mushrooms. Park and Sanders[131] sampled outdoor market produce and detected campylobacters in spinach, lettuce, radish, green onions, parsley, and potatoes at 1.6% to 3.3% incidence. No campylobacters could be detected following minimal sanitization.

40.5.2 Incidence in Poultry and Poultry Products

Chickens, turkeys, domestic ducks, pheasants, guinea fowl, emu, and ostrich have all been associated with high rates of colonization by *C. jejuni*.[74,108,118,126,166] Campylobacters have been isolated also from hawks, parrots, and pigeons.[34,126] By far the most important avian species associated with human exposure to *C. jejuni* is the chicken.[42] Most of the 7 billion birds produced within the US in 1996 were raised under intensive management conditions,[183] though free-range birds have also been shown to be colonized by *C. jejuni* at a similar rate.[183] Cloacal swabs were taken from poultry raised under intensive and free-range management respectively. Rates of isolation appear similar among intensive-reared and free-ranged bird flocks.[5] *Campylobacter jejuni* usually is not isolated from the hatchery, nor is it typically isolated from the production environment during the first two weeks after chicks are placed; however, by the third or fourth week of production, most flocks are contaminated to some extent and the organism eventually spreads to almost all members of the flock. The intestinal tract of chickens may harbor up to 10^7 cfu *C. jejuni* g^{-1} without any apparent pathological consequences for the host.[168] During processing, poultry carcasses may become contaminated by the release of intestinal contents;[121] most raw poultry products are contaminated with *C. jejuni*.[21,166]

40.5.3 Incidence in Pigs and Pig Products

Campylobacters are often found in the intestinal tract of swine and are sometimes associated with enteric disease in pigs;[21,121] most isolates are *Campylobacter coli* rather than *C. jejuni*. Swine carcasses are more frequently contaminated with campylobacters than either cattle or sheep carcasses.[60] However, the reported rate of contamination from swine carcasses varies widely from 2.9% in Poland[101] to 16.9% in Canada[102] and 95% in Sweden.[177]

40.5.4 Incidence in Cattle, Beef, and Cow's Milk

Cattle have been implicated in numerous outbreaks of human campylobacteriosis, primarily associated with the consumption of unpasteurized milk.[14,141,147] Milk may become contaminated by exposure to fecal material[186] or through mastitic infection.[116,125] Cattle as a meat source is not strongly implicated in transmission of campylobacters to humans. Although healthy cattle may excrete *C. jejuni* in their feces and carcasses may become contaminated during processing, this occurs infrequently and the numbers of contaminants are low.[121] Stern et al.[170] analyzed samples of beef liver, beef cheek meat, and ground beef using presence/absence testing (minimum level of detection was 10 cells) and found that only about 5% of red meat samples from retail outlets around the US were positive for campylobacter. An earlier study,[182] which employed both enrichment and direct-plating techniques, found only 1.6% of 6,169 meat samples positive for the organism.

40.5.5 Incidence in Sheep and Sheep Products

Campylobacter jejuni lives as a benign commensal organism in the intestinal tract of some sheep[21] but may cause infectious abortion in others.[161] Isolation of the organism from the intestinal contents of sheep is common and contamination of carcasses can occur.

40.5.6 Incidence in Domestic Pets and Other Animals

Domestic pets such as dogs and cats may harbor campylobacters in their intestinal tracts and may serve as important reservoirs for human infection. Moreno et al.[115] isolated campylobacters from 66% of healthy cats and 34% of healthy dogs. Campylobacter isolation rates are higher among young puppies and kittens than in mature dogs and cats,[20] and the pathogens are isolated more frequently from kennel populations than from household pets.[26] More exotic pets such as the golden Syrian hamster may harbor campylobacters as may numerous zoo animal species including ungulates, felines, and primates.[108] Wild rodents[57] and flies[151] may also carry campylobacters and could be important vectors in contamination of poultry houses. Campylobacters are isolated infrequently from reptiles probably because of the high (42° C) optimum growth temperature for the microorganism.[108]

40.6 EPIDEMIOLOGY

The epidemiology of human campylobacter infection reflects a pattern of numerous sporadic, individual infections and relatively infrequent large outbreaks. In sporadic cases of campylobacteriosis occurring among members of a health maintenance organization in Seattle, about 50% were accounted for by poultry[73] and could be traced to the mishandling of contaminated poultry products. Among university students in Georgia, 70% of cases were accounted for by eating chicken, often undercooked or raw.[178] In another study in Colorado, handling raw chicken, as opposed to eating it, emerged as a risk factor.[77] Poor kitchen hygiene may also play a role, and in one study the risk of infection was inversely associated with the frequency of using soap to clean the kitchen cutting board.[178] Further studies in many countries have shown that *C. jejuni* serogroups isolated from poultry sources are the same as some of the predominant serogroups isolated from humans.[7,8,61,90,91,117,123,142,150,152] *Campylobacter* isolates from swine (typically *C. coli*) do not belong to serotypes found in humans; therefore, pigs do not appear to be a major vector for human campylobacteriosis. Although as noted above, cattle are an important source of milkborne campylobacteriosis, meat products are not strongly implicated.[166]

Skirrow[158] and Blaser[13] reported isolation rates of *C. jejuni* of 7.1% and 5.1%, respectively, from the stools of patients with gastroenteritis. Prior to these early reports, clinical laboratories responsible for isolation and identification of causative agents of foodborne illness did not culture stools at 42° C under reduced atmospheric conditions (microaerobic atmospheres) and therefore failed to detect campylobacters.

In many countries, including the Netherlands, the United Kingdom (UK), France, Italy, Sweden, the US, and Australia, campylobacters are the main cause of acute infectious diarrhea. The estimated annual incidence for campylobacteriosis in the US in 1988 was approximately 1,000 cases per 100,000 population[179] and is close to the annual estimated infection rate in England and Wales of 1.1%.[96] In the US in 1996, FoodNet began collecting population-based active surveillance data on culture-confirmed cases of seven foodborne diseases, including campylobacteriosis. Incidence rates in stool specimens or specimens from normally sterile sites were highest for campylobacteriosis at 25 per 100,000 population; rates ranged from 14 (Georgia) to 58 (California).[36] In 1996, the British Public Health Laboratory Service Communicable Disease Surveillance Centre received a total of just over 43,000 laboratory reports of campylobacteriosis; numbers of reports received in the first 20 weeks of 1997 were 7% higher than in the same period of 1996.[109] A study conducted in the Netherlands in 1992 and 1993 yielded an annual incidence of campylobacter infection of 69 cases per 100,000 people.[65] However, the true incidence of campylobacteriosis in the general population is likely to be much higher, because only a small proportion of people with gastrointestinal complaints consult a physician, as discussed in Chapter 37. In developed countries, the demographic incidence of campylobacteriosis usually follows a bimodal distribution with infection occurring most frequently in children under the age of 4 years and in young adults between 20 and 30 years of age.[179] In developing countries *Campylobacter* infection occurs most frequently in children under 5 years of age with a prevalence of infection much greater than in developed countries.[21] For children aged less than 1 year, rates per 100,000 population for campylobacteriosis ranged from 25 (Georgia) to 193 (California) in the US in 1996.[36]

When outbreaks of campylobacteriosis occur, they are most often associated with consumption of raw, unpasteurized milk or milk that has been contaminated postpasteurization.[88] Outbreaks generally demonstrate a bimodal distribution with peaks in May and October.[121] By contrast, the far more numerous sporadic cases of campylobacteriosis are associated with consumption of foods of animal origin, and the seasonal incidence of infections typically demonstrates a summertime peak[30] suggesting different epidemiological characteristics for outbreak-associated and sporadic cases. The Centers for Disease Control and Prevention (CDC) Foodborne Diseases Active Surveillance Network reported that 35% of the sporadic cases occurred during the summer months (June-August).[36]

The importance of food handlers in transmission of *Campylobacter* infection has not been fully defined, nor has the role of person-to-person contact. Two cooks who were asymptomatic excreters of *C. jejuni* did not transmit the pathogen to prepared foods.[122] However, campylobacters were isolated from the hands of a food handler suffering from campylobacteriosis.[15] Person-to-person transmission of *Campylobacter* can occur and studies in Canada[128] and England[88] demonstrated that transmission from ill children to family members occurred frequently. By contrast, in two outbreaks among Japanese children secondary transmission was not observed.[84,191]

Perinatal transmission of *Campylobacter* infection is possible, although rare. Pregnant women infected with *Campylobacter*-associated bacteremia may pass on a severe systemic infection to the fetus.[21] A case of maternal sepsis caused by *C. jejuni* was reported in Switzerland, in which the etiologic agent was confirmed using molecular methods on placental tissues.[113] It is also possible for neonates to acquire infection during or shortly after birth. Anders et al.[6] reported seven cases of *Campylobacter* enteritis in neonates over a 2-year period in Denver in which symptoms began from 2 to 11 days after birth.

Direct animal contact also is responsible for transmission of *Campylobacter* infections to some humans. Among people living in farming communities, a higher incidence of campylobacteriosis is observed in those who have occupational exposure to poultry and cattle.[87] Likewise, serological surveys demonstrated that people employed in poultry and red meat plants have a significantly higher rate of seropositivity to *Campylobacter* antigens than rural field laborers.[87] Contact with household pets such as cats and dogs may also be responsible for transmission of *Campylobacter* infection, as similar bio- or serotypes are frequently isolated from humans and animals.[19] Newly acquired puppies and kittens are generally a greater risk factor than are older adult animals, especially if the pet develops diarrhea.[122] Studies conducted in England reported 3 cases of *Campylobacter* infection associated with cats and 97 cases associated with dogs.[160]

40.7 FACTORS AFFECTING SURVIVAL, GROWTH, AND CONTAMINATION OF FOODS

Campylobacters do not grow below 30° C or in the presence of atmospheric levels of oxygen. Therefore, growth of campylobacters in food is not a consideration, though survival of the organism on contaminated foods is a concern in view of the low infective dose.[47] Factors that affect the survival of campylobacters include: temperature (heating, refrigeration, and/or freezing), pH, sensitivity to sodium chloride (NaCl) or spices, atmospheric conditions, irradiation, and food preparation.[83] Viable, nonculturable campylobacters have been reported.[149] Contamination of poultry meat is thought to originate from the intestinal tract and then spread to the meat during transport and processing.[118] Generally, counts are at their highest immediately following evisceration, though for contaminated broiler carcasses $10^{2\text{-}3}$ cfu campylobacter ml^{-1} of carcass rinse are common when sampled from the chiller. Prior to further processing or transport to a retail market, carcasses and other poultry products may be stored under chilled or frozen conditions; although the organism survives frozen storage, survival is generally better under chilled storage. Hanninen[70] studied survival of four strains of *C. jejuni/coli* on frozen broiler carcasses. During 12 weeks of storage at –20° C counts decreased from an initial inoculum of between 10^3 and 10^5 g^{-1} to between $10^{0.5}$ and 10^2 g^{-1}. Oosterom et al.[124] reported that freezing affected *C. jejuni* viability only during the first few hours, and after an initial drop in numbers, it survived well on chicken carcasses and chicken livers stored at –20° C for more than 64 and 84 days, respectively. Stern et al.[170] collected approximately 800 fresh and frozen meat and poultry meat samples and analyzed them for *C. jejuni*. Overall fresh versus frozen isolation rates were 12.1% and 2.3%; for poultry samples, these were 21.3% and 7.5%, respectively. In another study, in the same year, Stern et al.[170] compared isolation rates from naturally contaminated carcasses (two groups of 15 broilers) before and after applying a freeze-thaw cycle. By direct plating, the percentage of positive carcasses decreased from 95% to 20% due to freeze-thaw injury (–15° C for 14 days); by enrichment, detection decreased from 90% to 53%.[172] Selective enrichment is essential for isolation of the organism from the frozen carcasses as cells of *C. jejuni* are injured by freezing.[144] Beuchat[10] demonstrated the survival of four of five strains of *C. jejuni* at –18° C for 12 months. He found that direct plating was superior to a most probable number (MPN) technique but that enrichment in Doyle and Roman's enrichment broth resulted in the highest level of detection. Stern[167] stored uninoculated carcasses (initial level of contamination, 6.3 x 10^5) at 4° C and sampled them at 0, 1, 3, 7, and 10 days. Using an enrichment method, detection rates of 86%, 88.75%, 81.25%, 80%, and 31.43% were reported, respectively; by direct plating, the organism was not detected after 10 days' storage.

Campylobacter jejuni grows at temperatures between 30° C and 45° C, with optimum growth at 42° C to 43° C;[173] death is more rapid at 25° C than at either 4° C or 30° C.[12,48] Heat injury of *C. jejuni* occurs at 46° C and thermal inactivation occurs at 48° C. Doyle and Roman[51] determined rates of thermal inactivation for five strains of *C. jejuni* in skim milk. At 48° C, *D*-values ranged from 7.2 to 12.8 minutes while at 55° C they ranged from 0.74 to 1.00 minutes. *D*-values for *C. jejuni* were greater in ground chicken meat than in peptone medium.[12] Thus at 49° C, 51° C, 53° C, 55° C, and 57° C, *D*-values in peptone were 15.2, 4.90, 1.71, 0.64, and 0.25 minutes, respectively, while in ground chicken, *D*-values were 20.5, 8.77, 4.85, 2.12, and 0.72 minutes, respectively. *D*-values for inactivation of campylobacters in ground beef ranged from 5.9 to 6.3 minutes at 40° C and from 2 to 21 seconds at 58° C, and in lamb meat *D*-values at 50° C ranged from 5.9 to 13.3 minutes and at 60° C from 12.5 to 15.8 seconds.[99] These data indicate that ordinary cooking temperatures should be sufficient to destroy campylobacters contaminating poultry and other meats.

Optimal growth of *C. jejuni* occurs at pH 6.5 to 7.5, with good growth at pH 5.5 to 8.0, and no growth below pH 4.9.[48] At pH 3.0 to 4.5 the rate of cell death decreased with decrease in temperature. Campylobacters are highly sensitive to NaCl (optimum growth concentration is 0.5%); sensitivity to NaCl is also temperature dependent.[50] Thus, at 4° C, *C. jejuni* was sensitive to 1.0% or more NaCl but the rate of death at this temperature was much less than that which occurred at 25° C; a 3 $\log_{10}$ decrease of cells occurred in the presence of 4.5% NaCl, after 1.25 to 2.1 days at 25° C, but a similar reduction at 4° C took about 2 weeks at the same salt concentration. Two strains of *C. jejuni* and *C. coli* tolerated more NaCl at 35° C than at 30° C.[71] Abram and Potter[2] investigated survival of *C. jejuni* in ground chicken, beef, and cod with 0%, 1%, and 2% added NaCl at –18° C, 6° C, and 10° C over a 5-day period. The survival of *C. jejuni* during frozen storage was not affected by NaCl levels tested, but at 6° C and 10° C, in both raw and cooked chicken and beef, significant decreases in cell numbers occurred with increasing NaCl concentration. Substantial decreases in cell numbers also occurred in refrigerated raw cod but there was less decrease in the cooked fish; numbers in raw cod decreased significantly with increasing NaCl concentration and the decline was more pronounced during storage at 10° C than at 6° C. These data suggest that even moderate levels of NaCl in foods can decrease *C. jejuni* survival. Deibel and Banwart[41] studied the effects of various concentrations of oregano, sage, and ground cloves on the growth of *C. jejuni* in a liquid growth medium incubated at 4° C, 25° C, and 42° C for up to 48 hours. At 42° C, growth occurred at the highest concentrations of spice tested, after 16 hours. At 25° C, greater than a 3 $\log_{10}$ decrease in colony forming units was observed in the suspensions containing sage or oregano, with sage showing the greatest effect. At 4° C, a less than 1 $\log_{10}$ reduction was observed for all concentrations of spice tested. Koidis et al.[100] determined the inhibitory effect of garlic, onion, black pepper, and oregano on survival of *C. jejuni* in a nutrient broth stored at 4° C for 12 days. At the highest concentration tested, a 3.9 $\log_{10}$ decrease was noted for the broth containing onion, while 3.0 $\log_{10}$ reductions below the respective controls (nutrient broth alone) were noted for broths containing the maximum concentrations of garlic, pepper, and oregano tested. Clearly, survivability is affected by the presence of spices. However, given the length of time required for substantial reductions in numbers to occur and the levels of spices tested, the practical application of the effect is questionable. Some effect may occur on the surface of a spiced meat product, where exposure to drying and oxygen occur, but these conditions alone are lethal to campylobacters.[51]

Outbreaks of campylobacteriosis from the consumption of contaminated milk have been documented;[16,54] therefore, survival of campylobacters in milk and milk products is of public health concern. The ability of campylobacters to survive heat treatment in milk has been evaluated.[63] The legal minimum requirement for pasteurization of milk in most countries is heating at a temperature of at least 72° C for not less than 16 seconds (see Chapter 22). Properly controlled pasteurization at 72° C inactivated campylobacters inoculated into milk, reducing counts from up to 10^6 ml^{-1} before pasteurization to undetectable levels after pasteurization.[63] In the case of one human isolate inoculated into milk at an initial concentration of 10^6 ml^{-1}, survivors were detected after heating at 72° C for 10 seconds but not after heating for 20 seconds. Variable rates of die-off in milk have been reported[48] with one strain showing only a 2 $\log_{10}$ decrease in viable cells after 14 days of refrigerated storage and another showing a greater than 10^6 log decrease after only 7 days at the same temperature; inactivation corresponded to increases in milk aerobic plate count and a decrease in milk pH. Ehlers et al.[53] inoculated milk to be used in the manufacture of cheddar cheese with 10^2 to 10^6 cells ml^{-1} of *C. jejuni* and found no viable cells in the curd after 30 to 60 days of curing.

A number of countries permit the use of gamma irradiation as a means of preserving foods, and the use of 3.0 kGy applied to poultry, specifically to control levels of salmonellae, was approved in the US in 1990.[56] Clavero et al.[37] inoculated raw ground beef patties with *C. jejuni* and subjected them to gamma irradiation treatment (^{60}Co) at varying doses. Effects of fat, freezing, and refrigerated temperatures were investigated. Regardless of fat level or temperature during inactivation, a 2.5 kGy dose inactivated $10^{10.6}$ *C. jejuni* g^{-1} of beef. Patterson[132] investigated the sensitivity of *Campylobacter jejuni*, *C. coli*, and *C. lari* to irradiation in poultry meat; D_{10} values obtained ranged from 0.12 to 0.25 kGy. There was no significant difference in the radiation sensitivity between species and between strains of the same species. Collins et al.[39] compared the ability of *C. jejuni* and *Arcobacter butzleri* to survive irradiation under vacuum in ground pork; D_{10} values for *A. butzleri* and *C. jejuni* were 0.27 kGy and 0.19 kGy, respectively. Based on these values, they predicted a 5 log decrease in *A. butzleri* cells and a 7 $\log_{10}$ reduction in *C. jejuni* cells following a 1.5 kGy treatment. Enteropathogenic *Campylobacter* is more sensitive to irradiation than either *Listeria monocytogenes* or *Salmonella*, and treatments sufficient to eliminate these pathogens from poultry carcasses would also eliminate campylobacters.

The survival of *Campylobacter* in foods is affected by the atmosphere. Blankenship and Craven[12] reported an increase in survival in chicken drumsticks when stored in carbon dioxide (CO_2) compared with an ambient atmosphere. After storage for 24 days, a 2 $\log_{10}$ decline in cell numbers was noted in the air-stored compared with the CO_2-stored meat. However, Wesley and Stadelman[188] evaluated the effect of CO_2 environment on 50 broiler carcasses over a 9-day period and reported no effect due to the modified atmosphere. The discrepancy between these two reports may be due to differences in the time for which samples were stored (9 vs. 24 days). In fresh beef samples inoculated with *C. jejuni* and then stored in three atmospheres (100% N_2; 20% CO_2 + 80% N_2; 5% O_2 + 10% CO_2 + 85% N_2) at 20° C or 4° C for 2, 4, and 25 days, survival at 4° C was similar for all three of the modified atmospheres, but the highest survival was in 100% N_2. Stern et al.[171] reported no significant difference in survival of *C. jejuni* in broiler chickens, when vacuum-packed or wrapped in oxygen-permeable, polyvinyl chloride film. However, vacuum-packed red carcass meat samples, stored refrigerated for 1 week, gave a significantly ($P<0.01$) higher rate of recovery of indigenous campylobacters than oxygen-permeable film-wrapped samples (19/63 vs. 7/63). The survival of *C. jejuni*, inoculated onto turkey roll and packed under CO_2, increased as the concentration of CO_2 was increased, at both 4° C and 21° C storage temperatures.[139] Grigoriadis et al.[68] investigated storage of hamburgers in 100% air, 100% CO_2, and 100% N_2 atmospheres at 4° C. An atmosphere of 100% air was the most toxic to the *C. jejuni* strains tested.

As early as 1983, Hopkins and Scott[77] found a correlation between handling raw chicken and incidence of sporadic campylobacteriosis. In a 2-year study to evaluate the prevalence of indicator bacteria and specific pathogens in 10 normal kitchens in the US, *Campylobacter* was isolated from the sinks of two houses, indicating that the organism can survive in the environment of a kitchen.[89] Acuff et al.[4] investigated washing procedures used for contaminated utensils (knives and cutting boards) and by food handlers for washing their hands, for their effectiveness at destroying *C. jejuni* after use in the handling and preparation of inoculated turkey thighs. Washing of utensils with water and detergent, either by hand or in a dishwasher, removed *C. jejuni* except from wooden cutting boards washed by hand. However, hand washing procedures, unless thoroughly applied, may not ensure complete removal of the organism from contaminated hands. *C. jejuni* cells survived better on wooden than plastic surfaces because of the physical structure of the wood.[25]

40.8 PRINCIPLES OF DETECTION, ISOLATION, IDENTIFICATION, AND TYPING

40.8.1 Detection, Isolation, and Identification

With the possible exception of raw poultry, which may harbor sufficiently large numbers of *C. jejuni* to enable their detection and enumeration by direct plating, most products require the use of enrichment methods to detect the small numbers of campylobacters that may be present. Some of these may be injured requiring particular conditions for their recovery.[129] Proper sample care, a large sample size, and the use of a selective enrichment broth, proper incubation conditions (including the correct atmosphere and temperature), and the plating of enrichments on to suitable selective plating media are essential for the reliable isolation of campylobacters from foods.[173]

Because of the fragility of campylobacters, food samples must be held under suitable conditions and processed rapidly to ensure optimum isolation. Thus food samples for analysis should be protected from exposure to air, transported under refrigeration or packed in ice (but must not be frozen), and analyzed as soon as possible after the collection of the sample.

The characteristics of campylobacters that make culture most difficult are their microaerophilic and capnophilic (CO_2-loving) nature. Oxygen at atmospheric concentration (21%) is toxic to campylobacters, which prefer only 5% oxygen and about 10% CO_2. Microaerobic conditions for growth of campylobacters can be achieved by a variety of methods including application of the Fortner principle[95] or atmosphere generating systems and gas exchange using pressurized gas cylinders to flush and fill anaerobe jars or sealable plastic bags.

Many different enrichment broths[24,49,130] and selective plating media[14,23,175] have been developed for recovery of campylobacters from foods. Most of these media use a basal medium consisting of beef extract, peptone, yeast extract, and NaCl to which supplements are added such as lysed or laked horse or sheep blood; charcoal is used in place of blood in some plating media. The aerotolerance of campylobacters in food samples can be optimized for improved recovery of the organism by supplementing the growth media with 0.025% each of ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP supplement).[62] The selectivity of the enrichment media is increased by the addition of various antibiotics including amphotericin B, cefoperazone, cycloheximide, polymixin B, rifampicin, trimethoprim lactate, and vancomycin.

Sampling procedures are similar to those used for microbiological analysis of foods for other pathogens and usually involve either rinsing or surface swabbing techniques. The surface rinse technique is especially useful for poultry carcasses or other moderately large pieces of food. The food sample is aseptically placed in a sterile plastic bag and 100 to 250 ml of brucella broth, buffered peptone water, or other suitable diluent is added. The sample is rinsed by shaking and massaging. The rinse suspension may then be filtered, if necessary, through layers of cheesecloth to remove particulate matter. The suspension may then be swabbed directly onto *Campylobacter* selective agar or serial dilutions may be plated if enumeration is desired. Alternatively, a portion of the rinse suspension may be mixed with an equal volume of double strength selective enrichment broth. This inoculated enrichment broth can be incubated directly and used for qualitative determination of *Campylobacter* presence or it can be further diluted and used to inoculate further tubes of media for quantitative estimation by the MPN technique (Chapter 60). If very small numbers of *Campylobacter* are expected, the original rinse suspension may be pelleted by centrifugation at 16,000 × g for 10 min. The supernatant may be discarded and the pellet resuspended in a lesser volume of about 2 to 5 ml of enrichment broth. Incubation usually is carried out under microaerobic conditions (eg, 5% O_2, 10% CO_2, 85% N_2) at 42° C for 24 to 48 hours with agitation. Recovery of injured campylobacters is facilitated by initial incubation at 37° C for 4 hours, at which time antibiotic supplements are added and the incubation temperature is increased to 42° C.[80,82,173]

Isolation, identification, and confirmation of *Campylobacter* isolates by cultural methods require plating enrichment broth samples after incubation onto selective agar plates. Typical colonies on selective agar media appear smooth, convex, and glistening with a distinct edge or flat, shiny, translucent, and spreading with an irregular edge. They are colorless to grayish or light cream and may range from pinpoint to 4 to 5 mm in diameter.[173] Growth of distinct colonies is facilitated by drying plates prior to inoculation and including in plate containers filter paper strips to which four drops of glycerol have been added.[165] Presumptive *Campylobacter* isolates can be observed using dark-field or phase-contrast microscopy for characteristic morphology and motility;[173] however, cells from cultures older than about 24 hours may appear coccoidal and nonmotile. Biochemical and growth parameter tests are used to further confirm an isolate as *Campylobacter*.[136] Although separation of *C. jejuni* and *C. coli* isolates may not be necessary in a food microbiology laboratory, the two strains may be separated by the hippurate hydrolysis test; *C. jejuni* is positive.[160]

Colonies of *Campylobacter* can be identified by commercially available latex agglutination tests such as those marketed by Integrated Diagnostics, Inc., Baltimore, MD; Mercia Diagnostics, Shalford, UK; and Becton Dickinson Microbiology Systems, Cincinnati, OH. Enzyme-linked fluorescent assay (ELFA) systems such as the VIDAS system (bioMerieux Vitek, Hazelwood, MO) provide fully automated qualitative detection of *Campylobacter* from a 24- to 48-hour enriched sample in about 70 minutes. Immunomagnetic separation methods coupled with polymerase chain reaction (PCR)-based detection systems are being developed for detection of *Campylobacter* from food samples.[44,127]

40.8.2 Typing Schemes

Typing of *Campylobacter* strains is particularly needed for epidemiological purposes. Typing methods can be divided into two main categories: those based on phenotypic characteristics such as heat-stable or heat-labile (HL) antigens[106,137] and those based on genotyping. Included in this latter category are restriction fragment length polymorphism (RFLP) of selected genomes such as done with ribotyping of other genomes,[119] bacterial restriction endonuclease DNA analysis (BRENDA), and pulsed field gel electrophoresis.

Phenotype-based Schemes

The most well developed typing schemes used in epidemiological studies are those based on the use of the soluble, thermostable lipopolysaccharide antigens that can be used in a passive hemoagglutination test,[103] and the use of thermolabile flagella antigens that can be used in a slide agglutination test.[106] More recently, phage typing schemes have been developed. Thus Grajewski et al.[66] originally described a set of 14 phage types that discriminated about 90% of the 255 human isolates tested. The number of phage types was expanded by Kaijser and Falsen,[97] who were able to discriminate 78% of the 301 isolates tested; a new phage typing scheme that claimed better discrimination and typability of campylobacters was described by Salama et al.[156] However, it is recommended that phage typing is used together with another typing method to discriminate confidently between isolates.

A further phenotypic assay involves the analysis of the OMP of campylobacters by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Although good discrimination is possible, limitations of SDS-PAGE to discriminate between isolates have been reported also.[18,43,107] In addition, considerable technician time is required for such analyses, and interlaboratory reproducibility has been poor.

A biotyping scheme that is commercially available (MAST, Camp-BID, Mast Diagnostics, Merseyside, UK) distinguishes between the four enteropathogenic *Campylobacter* spp. and subdivides these into 55 groups.[22] The scheme is based on antibiotic-resistance patterns and limited biochemical assays and provides a four-digit code that indicates the species and subgrouping. It could be useful for laboratories not in need of the ultimate discriminatory capability by allowing tracking abilities with simplified technology and limited costs.

Genotype-based Schemes

A comparison of 10 methods for subgrouping *Campylobacter* strains based on genotyping was published in 1991.[134] The genotyping methods studied included multilocus enzyme electrophoresis (MEE), BRENDA, restriction digests of selected bacterial chromosomal DNA (ribotyping), and plasmid profile analysis; these methods are fully described in Chapter 64. The authors concluded that MEE, BRENDA, and ribotyping were the most sensitive methods and were capable of identifying 9 types among 22 strains. The authors observed that MEE and ribotyping had several advantages over the other methods because they measured relatively stable and significant chromosomal differences and were applicable to other species and genera. Hernandez et al.[75] analyzed 39 strains of campylobacters by ribotyping and confirmed these conclusions. Modification to the method by Patton et al.[134] employed use of the HaeIII enzyme to digest the isolated chromosomal DNA; on electrophoresis of the digest product, they were able to distinguish 32 different band profiles that allowed discrimination between the strains analyzed. Each ribotyping pattern comprised between 3 and 11 fragments of 1 to 10 kb.

A further genotyping scheme for *Campylobacter* has been described that entails probing the flagellin gene, *flaA*, for differences or similarities between strains.[119] The method uses PCR and RFLP.

40.9 CONTROL IN POULTRY OPERATIONS

Despite substantial effort by international researchers, contamination of domestic meat animals, especially poultry, continues to be a worldwide problem. When contaminated animals are presented for slaughter, the opportunity for spread of zoonotic pathogens during dressing of the carcass (particularly during defeathering and evisceration) is substantial, even under the best of conditions. Stern et al.[169] demonstrated that chicken carcasses become more contaminated with *C. jejuni* when broilers are moved from the farm to the processing plant. One hundred birds from 10 separate flocks were sampled at the farm by whole carcass rinse and another hundred carcasses were analyzed following transportation but prior to processing. Prior to transportation, an average of log cfu of 3.66 campylobacters per carcass were identified; following transportation, carcasses averaged log cfu of 7.11 of campylobacters per carcass. Prevalence was also affected: before transportation, only 6 of the 10 flocks were positive for campylobacters as determined by whole carcass rinse, while 9 were positive after transport. Procedures required to remove or kill this pathogen once it has become attached to carcasses and derived meat products would be likely to decrease the organoleptic quality of the meat. However, if means were developed to reduce the incidence campylobacters in the chicken gastrointestinal tract, then processed carcasses with reduced levels of the pathogen could be achieved. Concomitantly, the reduced incidence and level of campylobacter-contaminated poultry products would reduce consumer exposure and should result in diminished frequency of the human disease. The means whereby campylobacters are introduced into a flock of chickens has been difficult to determine and is still disputed, but once in the flock, the gastrointestinal tracts of most birds soon become colonized.[55,81,94,105,157,184] Campylobacters are not an essential part of normal poultry flora because birds can be brought to market without colonization. Pearson et al.[135] suggest the possibility of vertical spread of the organism from the hatchery; however, this remains controversial. Campylobacters normally cause no pathology or change in growth of the chicken; therefore, it is more accurate to refer to the habitation of the chicken intestinal tract by these organisms as a "colonization" rather than an infection. The primary incentive to poultry producers to preventing colonization, therefore, is to increase the human health attributes of the final product.

In one study of broilers at slaughter, 79% of the birds were colonized with campylobacters.[140] Flocks apparently vary in prevalence of colonization from none to 100%;[86,174] however, a prevalence of less than 100% could be due to the timing of sampling relative to the time when the organism was introduced to the flock. Not only is the prevalence of campylobacters in poultry high, but the levels of the organism on carcasses of fully processed chickens have been reported as up to 1.5×10^6 cfu per chicken.[76,85]

Dolby and Newell[45] reported that by intraperitoneal vaccination of female mice with a strain of *C. jejuni* before mating, the dams conferred immunity to their young, demonstrated by oral challenge 4 to 6 days after birth with the homologous strain; they were unable to demonstrate protection against a heterologous strain. Abimiku and Dolby[1] confirmed these observations and also reported that the mouse pups required the consumption of immune milk at and after the time of challenge to resist colonization by *C. jejuni*. These two reports strongly suggest the possibilities of vaccine administration to prevent the colonization by *Campylobacter*.

In the UK, epidemiological investigations linked many sporadic cases of *Campylobacter* infection to chickens from one farm.[135] Water was suggested to be the principal source for the spread of the organism on the farm, based on detection by filtration and fluorescent antibody techniques. It was suggested also that scrubbing the water storage tanks with quaternary ammonium compound disinfectant diminished the presence of campylobacter in chickens.

Rollins and Colwell[149] demonstrated that *C. jejuni* can exist in a viable, nonculturable form (VBNC). Cells in this form were able to take up radio-labeled amino acids and maintained intact outer membranes, but were unable to produce colony-forming-units on selective campylobacter media. Stern et al.[172] grew six poultry isolates of *C. jejuni* overnight on agar plates, suspended the growth from these at 10^8 cells ml^{-1} in phosphate-buffered saline, and maintained the suspensions at 4° C; at weekly intervals, the number of viable organisms was determined on Brucella-FBP agar (a nonselective medium). When the organisms were no longer detectable by direct plating on Brucella-FBP agar (4 to 7 weeks), 50 ml of each of the original suspensions were cultured by enrichment and this enrichment procedure was repeated 2 weeks later. At neither time were any of the six strains successfully cultured. Once the VBNC status of the cultures was determined, day-old chicks (n = 11 to 20) were orally gavaged with 0.2 ml of each of the suspensions. The birds were placed in isolation units and segregated according to challenge culture. An additional isolation unit was used to house unchallenged control chicks. One week after challenge, the birds were sacrificed and the cecal contents were cultured directly on Campy-Cefex selective agar. Four of the *C. jejuni* strains colonized low numbers of the chickens (5 of 79), while the other two isolates did not (0 of 30). None of 18 control chicks were colonized. The original and recovered strains were analyzed by BRENDA and HL antigen typing; they were indistinguishable. The discovery of a colonizing but nonculturable form of *Campylobacter* provides a potential explanation for sources of the bacterium in broilers. The chicken-passaged, previously nonculturable materials yielded *Campylobacter* that were indistinguishable from the original isolate of *Campylobacter*.

40.10 CONCLUDING REMARKS

Although campylobacters are apparently fragile organisms easily killed by all commonly applied methods used to decontaminate foods, they, and in particular *C. jejuni*, are the leading cause of bacterially induced human gastroenteritis. A possible reason why such a fragile bacterium can cause so many cases of gastrointestinal illness is its low infective dose combined with its widespread occurrence, often in large numbers, on poultry carcasses, providing opportunity for postcooking recontamination of poultry and cross-contamination via utensils and working surfaces onto other foods. A terminal pasteurization step (eg, irradiation or heat pasteurization) applied under controlled conditions at the processing plant, seems to be the best means currently available of preventing campylobacteriosis. It is incumbent on the industry and researchers to continue to explore strategies that will reduce levels of consumer exposure. As this organism is incapable of multiplying on foods at temperatures below 30° C, controlling the colonization of the intestinal tract in farm animals (eg, by vaccination) or finding ways to limit carcass contamination could accomplish this ambitious goal.

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41 *Clostridium botulinum*

Barbara M. Lund and Michael W. Peck

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also nitrite, and refrigeration during salting; shelf-stable raw salted and salt-cured meats
41.9.6 Category 5. Shelf-stable products, *C. botulinum* controlled by reduced water activity, acidity, and nitrite; dry fermented sausages
41.9.7 Category 6. Perishable products, *C. botulinum* controlled mainly by refrigeration
41.9.8 Category 7. Perishable products, *C. botulinum* controlled mainly by sodium chloride and refrigeration, often in combination with nitrite, sometimes with other factors such as sorbate, benzoate, and nitrate; salted fish, lightly preserved and semipreserved fish products, hot-smoked fish, perishable, cooked, cured meats
41.9.9 Category 8. Perishable or shelf-stable products, *C. botulinum* controlled by mild heat treatment, reduced water activity, acidity, and refrigeration of some products; process cheese and process cheese spreads
41.9.10 Category 9. Perishable products, *C. botulinum* controlled by mild heat treatment and refrigeration; ready meals, "sous vide" foods
41.10 Concluding remarks
References

41.1 INTRODUCTION

The species *Clostridium botulinum* comprises a group of Gram-positive, spore-forming, anaerobic bacteria that form a powerful neurotoxin. Botulism in adult humans is caused by the consumption of food containing toxin produced by growth of *C. botulinum*. Sometimes the organism contaminates deep wounds where it multiplies and forms toxin, which spreads from the wound to cause the characteristic symptoms of botulism (wound botulism). Infant botulism, which usually affects infants less than 35 weeks old, is caused by ingestion of spores followed by establishment of the organism in the intestinal tract where it multiplies and forms toxin. Very rarely botulism associated with colonization of the gut has been reported in an adult who has undergone surgery for intestinal disease.[50,197]

C. botulinum was first isolated in 1897; subsequent isolates were shown to form neurotoxins that differed antigenically and were therefore differentiated into several types: A, B, C, D, E, F, and G. Based on their physiological properties, and later confirmed by their phylogenetic relationship, strains of *C. botulinum* were classified into four groups (Table 41–1); this grouping does not correspond with the differentiation according to the antigenicity of the neurotoxins. The use of a single species name for bacteria in Groups I to IV does not reflect the true phylogenetic relationships of these bacteria or their physiological properties.[54] It is retained because of the importance of these bacteria as pathogens in humans, animals, and birds, and the confusion that would be caused by renaming the bacteria in accordance with their phylogenetic and physiological similarity.[232]

Although many strains of *C. botulinum* produce toxin of a single antigenic type, some strains produce two types of toxin, usually a major amount of one toxin and a minor amount of the other.[128] Such strains have been designated Af (indicating the major and minor toxin respectively), Bf, Ab, and Ba. In 1979–1980, a case of infant botulism in New Mexico was attributed to an organism that produced type F toxin[145] and was subsequently identified as a strain of *C. baratii*[117]and two cases of infant botulism in Italy in 1984 were attributed to bacteria that resembled *C. butyricum* but formed type E toxin;[15,196] the identity of these organisms as *C. baratii* and *C. butyricum*, respectively was confirmed by study of their DNA relatedness.[309] In 1987 a further strain of *C. baratii* that formed type F toxin was isolated from an adult suffering type F botulism, probably caused by intestinal colonization,[197] and an outbreak of foodborne botulism in China in 1994, which affected six people, was attributed to a strain of *C. butyricum* that formed type E toxin.[202]

A history of developments in relation to botulism was outlined by Smith and Sugiyama[292] and Hauschild[127] and is summarized and updated in Exhibit 41–1. Early outbreaks associated with meat and poultry products were summarized by Tompkin.[327]

The term "botulism" is derived from the Latin *botulus* (sausage) and the name was given to a disease, reported mainly in Central Europe in the 1800s, that was generally associated with sausage consumption. *Clostridium botulinum* was first isolated by van Ermengem in 1897 from raw, salted ham that caused an outbreak of the disease in Belgium and from the spleen of a victim. Later the isolates were lost, but reports of the properties of the organism suggest that it would now be classified as nonproteolytic *C. botulinum* producing type B toxin. In early studies the name *C. botulinum* was used for nonproteolytic strains and *C. parabotulinum* for proteolytic strains. This distinction

Table 41–1 Groups of *Clostridium botulinum*, Toxins Formed and Main Species Affected

Group	*Toxins Formed*	*Main Species Affected*	*Examples of Main Food or Feed Implicated*
I (proteolytic)	A	Humans	Canned, low-acid foods (mainly home-canned)
	B	Humans Cattle; horses	Canned, low-acid foods (mainly home-canned) Hay or silage that may be contaminated by carcasses of small animals
	F	Humans	Very few cases reported
II (nonproteolytic)	B	Humans	Some outbreaks due to home-cured hams
	E	Humans Salmonid fish	Fish products Cannibalism of dead fish
	F	Humans	Very few cases reported
III	C (C_1 and C_2)	Birds, particularly waterbirds, also farmed chickens and pheasants	Carcasses of vertebrates and invertebrates
		Cattle, horses, mink, and other animals	Hay, silage, or poultry litter that may be contaminated with carcasses of small animals
	D	Cattle, sheep	Carcasses or bones of small animals
IV	G	Lack of evidence	

Source: Data from M.W. Eklund and V.R. Dowell, Jr., *Avian Botulism*, © 1987, Charles C Thomas Publisher, Ltd., and L.D.S. Smith and H. Sugiyama, *Botulism*, © 1988, Charles C Thomas Publisher, Ltd.

was based on the ability of the bacteria to digest cubes of coagulated egg white, cooked meat particles and casein; proteolytic and nonproteolytic strains showed a marked quantitative difference in this ability.[116] Subsequently the name *C. botulinum* was adopted for both of these groups and for Group III and later Group IV organisms, for which the name *C. argentinense* was proposed later.[310]

Human botulism worldwide is almost always caused by toxins of types A, B, and E.[124] Fewer than 10 incidents of foodborne type F botulism in humans have been documented, and there are reports of two incidents attributed to organisms producing type C toxin and one to an organism producing type D toxin.[124] Botulism occurs in many types of animals other than man and is usually due to Group III organisms producing type C or D toxin.[292] Proteolytic *C. botulinum* forming type B toxin has caused outbreaks of botulism in cattle and of toxicoinfectious botulism in horses, which are reported to be particularly susceptible to type B toxin. The majority of outbreaks of botulism in birds, particularly water birds, have been caused by Group III organisms producing type C toxin, but some outbreaks due to organisms forming type E toxin have been reported, probably due to the consumption of toxic fish.[77]

Since 1980 the medical use of purified botulinum toxin type A for treatment of hyperactive muscle disorders has been investigated; use of the toxin for certain types of treatment was licensed in the United States (US) in 1989 and in the United Kingdom (UK) in 1991.[237,271]

41.2 TAXONOMY AND PROPERTIES OF THE ORGANISMS

41.2.1 Taxonomy

Clostridium botulinum is differentiated into four groups on the basis of physiological characteristics that are shown, together with those of neurotoxin-forming strains of *C. butyricum* and *C. baratii*, in Table 41–2.

Both DNA-DNA hybridization studies and 16S-rRNA sequence analysis have shown that while strains within a phenotypic group are highly related, regardless of the toxin type produced, the four phenotypic groups correspond to four phylogenetically

Exhibit 41–1 Milestones in Relation to Botulism

Year	*Notes*
1793	Outbreak of botulism recorded in Wildbad associated with consumption of blood sausage. Thirteen people were ill and a further six died.
1815–1828	Kerner documented 234 cases of botulism predominantly associated with blood and liver sausage.
1895–1897	An outbreak of botulism in Belgium affected 23 of a group of 34 musicians; 3 died and 10 nearly died. The outbreak was associated with consumption of homemade ham. van Ermengem isolated a toxigenic bacterium (*Bacillus botulinus*) from the ham and from the spleen of a man who died. This was probably a nonproteolytic strain that produced type B toxin.
1904	Landman isolated a toxinogenic bacterium from salad, made with home-canned beans, that caused an outbreak of botulism in Darmstadt, Germany. The toxin was later designated type A.
1910	Leuchs reported that the above strains produced serologically distinct neurotoxins.
1919	Burke divided American isolates into those that produced type A and others that produced type B toxin. van Ermengem and Landman strains were no longer available for testing.
1922–1925	Organisms that caused a paralytic disease of chickens in the US and bulbar paralysis of cattle in Australia were designated as producing type C toxin.
1928–1929	An organism isolated from an outbreak of paralytic disease (lamsieke) in cattle in South Africa was shown to produce a toxin designated as type D.
1934–1937	Hazen in the US and Kushnir in Russia separately isolated *C. botulinum* from fish associated with human botulism.
1936–1937	Hazen's and Kushnir's strains were shown to produce an antigenically similar toxin designated type E.
1943	Wound botulism was first reported in the US.
1946	Lamanna crystallized type A toxin and showed that it was a protein.
1953	*C. botulinum* was defined as species designation for all bacteria known to form botulinum toxin, and therefore able to cause botulism in man or animals; the term *C. parabotulinum* was eliminated.
1958–1960	A new strain (proteolytic, Langeland) later shown to form type F toxin, was implicated in an outbreak of botulism that affected four people on the island of Langeland and was due to homemade liver paste.
1965–1966	Nonproteolytic strains that produced type F toxin were isolated from marine sediments and from fish.
1970	A bacterium described as producing as type G neurotoxin was isolated from soil in Argentina.
1970	A strain was described that produced two toxins (types A and F). Other strains that produce two toxins have been isolated since.
1976	Infant botulism was recognized. A case in 1975 was recognized retrospectively. Strains isolated were proteolytic and produced type A or type B toxin.
1980–1982	Infant botulism due to a bacterium producing type F toxin was reported; the bacterium was later identified as *C. baratii.*
1981	A strain of *C. botulinum* that formed type B as the major toxin and a minor amount of type A toxin was isolated from a case of infant botulism. A further isolate from a case of infant botulism produced type B as the major toxin and a minor amount of type F toxin.

continues

Exhibit 41–1 continued

Year	*Notes*
1986	*Clostridium butyricum* strains that produced type E botulinum toxin were isolated from cases of infant botulism.
1990	*C. botulinum* producing type C toxin was reported as the cause of infant botulism in Japan.[228]
1992	*C. botulinum* type B toxin was reported to be a zinc endopeptidase.[273]
1992	Clinical trial of Human Immune Globulin for treatment of infant botulism was started.[12]
1994	An outbreak of foodborne botulism in China was attributed to a strain of *C. butyricum* that produced type E toxin.[202]

Table 41–2 Physiological Properties of Bacteria That Form Botulinum Toxin

	C. botulinum Group					
	I	*II*	*III*	*IV (C. argentinense)*	*C. butyricum*	*C. baratii*
Toxin types	A,B,F	B,E,F	C,D	G	(E)	(F)
Proteolysis	+	–	–	+	–	–
Liquefaction of gelatin	+	+	+	+	–	–
Fermentation of:						
Glucose	+	+	+	–	+	+
Fructose	±	+	±	–	+	+
Mannose	–	+	+	–	+	+
Maltose	±	+	±	–	+	+
Sucrose	–	+	–	–	+	+
Trehalose	–	+	–	–	+	–
Lipase	+	+	+	–	–	–
Lecithinase	–	–	–	–	–	+
Metabolic acids[a]	A,iB,B,iV,PP	A,B	A,P,B	A,iB,B,iV,PA	A,B	A,B
Optimum growth temperature	30–40° C	25–37° C	40° C	37° C	30–37° C	30–45° C
Minimum growth temperature	10–12° C	3.0° C	15° C		~ 10° C	
Spore heat resistance (temp/D–value, min)[b]	121° C/ 0.21	82.2° C/ 0.4–2.4 min[c]; up to 231 min[d]	104° C/ 0.1–0.9	104° C/ 0.8–1.2		
Similar nontoxic organism	*C. sporogenes*	No species name assigned	*C. novyi*	*C. subterminale*	Typical *C. butyricum*	Typical *C. baratii*

Note: For biochemical reactions: +, all strains give positive reactions; –, all strains give negative reactions; ± = some strains give positive and others give negative reactions. Where temperatures are not given, they are not readily available in the literature.

[a] Metabolic acids: A, acetic; B, butyric; P, propionic; iB, isobutyric; iV, isovaleric; PP, phenylpropionic; PA, phenylacetic

[b] Heat resistance determined in phosphate buffer pH 7.0

[c] Heat resistance determined without lysozyme in the recovery medium

[d] Heat resistance determined with hen egg white lysozyme, 625 units ml^{-1}, in the plating medium

Source: Adapted with permission from C.L. Hatheway, *Clostridium Botulinum* and Other Clostridia that Produce Botulinum Neurotoxin, in *Clostridium Botulinum, Ecology and Control in Foods*, A.H.W. Hauschild and K.L. Dodds, eds., pp. 3–20, © 1993, by courtesy of Marcel Dekker, Inc.

distinct groups.[54] Strains from Groups I and II show a low relatedness even though they may produce the same toxin type. The distinction between Groups I to IV is strong enough to merit the creation of four species, were it not for the desire for practical purposes to retain the name *Clostridium botulinum* for these groups.

It is convenient to refer to strains of *C. botulinum* as "*C. botulinum* type A," etc. depending on the toxin formed, but it would be more correct to use the term "*C. botulinum* toxin-type A," as the "type" refers to the antigenic type of the toxin formed, not to properties of the organism itself. Because both proteolytic and nonproteolytic strains can form type B and type F toxins, in referring to a strain as type B or type F there is a need to make clear whether the strain belongs to Group I proteolytic or Group II nonproteolytic *C. botulinum.*

41.2.2 Properties of Bacteria That Form *Clostridium botulinum* Neurotoxin

The *C. botulinum* group of bacteria are anaerobic, motile, spore-forming rods 0.5 to 2.0 μm wide and 1.6 to 20 μm long.[42] Because botulism in humans is caused predominantly by Group I and Group II strains, this chapter will concentrate on these organisms. Group I organisms are actively proteolytic (ie, they are able to digest native proteins and rapidly break down particles of meat in meat media, cubes of coagulated egg white, and casein).[116] They also rapidly degrade the derived protein, gelatin. Group II organisms do not degrade these native proteins but liquefy gelatin, although less actively than Group I strains. Isobutyric acid, isovaleric acid, and phenylpropionic acid are formed as degradation products of the amino acids valine, isoleucine/leucine, and phenylalanine, respectively, by Group I but not Group II strains (Table 41–2).[42] The minimum temperature for growth of these bacteria is of major importance: Group I strains are unable to multiply at temperatures of 10° C and lower, whereas Group II strains can multiply at temperatures as low as 3.0 to 3.3° C.[79,80,112,276] The spores of Group I strains are much more heat-resistant than those of Group II strains (Section 41.7).

41.2.3 Properties of Spores of *Clostridium botulinum*

Studies of the physiology of sporulation have shown marked differences between Group I and Group II *C. botulinum.*[292] The spores of all groups are subterminal; those of Group I strains swell the sporangium markedly while those of Groups II and III strains do so to a lesser extent. The structure of the spores is similar essentially to that of other species of *Clostridium* and of *Bacillus.* In many cultural conditions, spores of Group I strains are retained within the sporangium for a prolonged period,[304] and even after harvesting and refrigerated storage in aqueous suspensions for several months the majority of mature, phase-bright spores may still be within a sporangium. In contrast, when spores of Group II strains are formed, the sporangium appears to be lysed within a few days, leaving the mature spore free of the sporangium.

Spores of some Group II, type E strains are contained within an exosporium that may be thin and membranous, thicker with a hexagonal pattern, or thicker and homogeneous.[143,144] In general, the function of the exosporium is unknown. Spores of some of these type E strains also carry a large number of appendages approximately 20 nm in diameter and up to 350 nm long, each appearing to be tubular with a hemispherical cap (Figure 41–1). These appendages do not appear to be associated with motility or attachment or to play a role in spore germination and outgrowth and have no known function.[292] Spores of the Group I strain 78A had a multilayered exosporium and were usually within a sporangium, whereas those of the Group II strain 17B were covered by an open-ended, sheath-like exosporium and usually were present as free spores.[304]

41.3 CHARACTERISTICS OF BOTULISM

Foodborne botulism is caused by ingestion of food containing toxin preformed during growth of the bacteria. Onset of symptoms usually occurs within 12 to 36 h but can occur within 2 h or after as long as 8 days.[292] Nausea and vomiting often precede other symptoms and probably are not caused by botulinum toxin but by other substances produced by *C. botulinum* during its growth in food.[292] The neurotoxin first affects neuromuscular junctions of the head and neck, preventing the passage of stimuli from motor nerves to muscles and resulting in double vision, inability to focus, drooping eyelids, thickness of speech, and inability to swallow or to speak clearly. As the disease progresses, there is increased muscle failure until muscles needed for breathing (the diaphragm and intercostal muscles) or the cardiac muscles fail and death follows. Foodborne botulism has been confused with other diseases including Guillain-Barré syndrome and my-

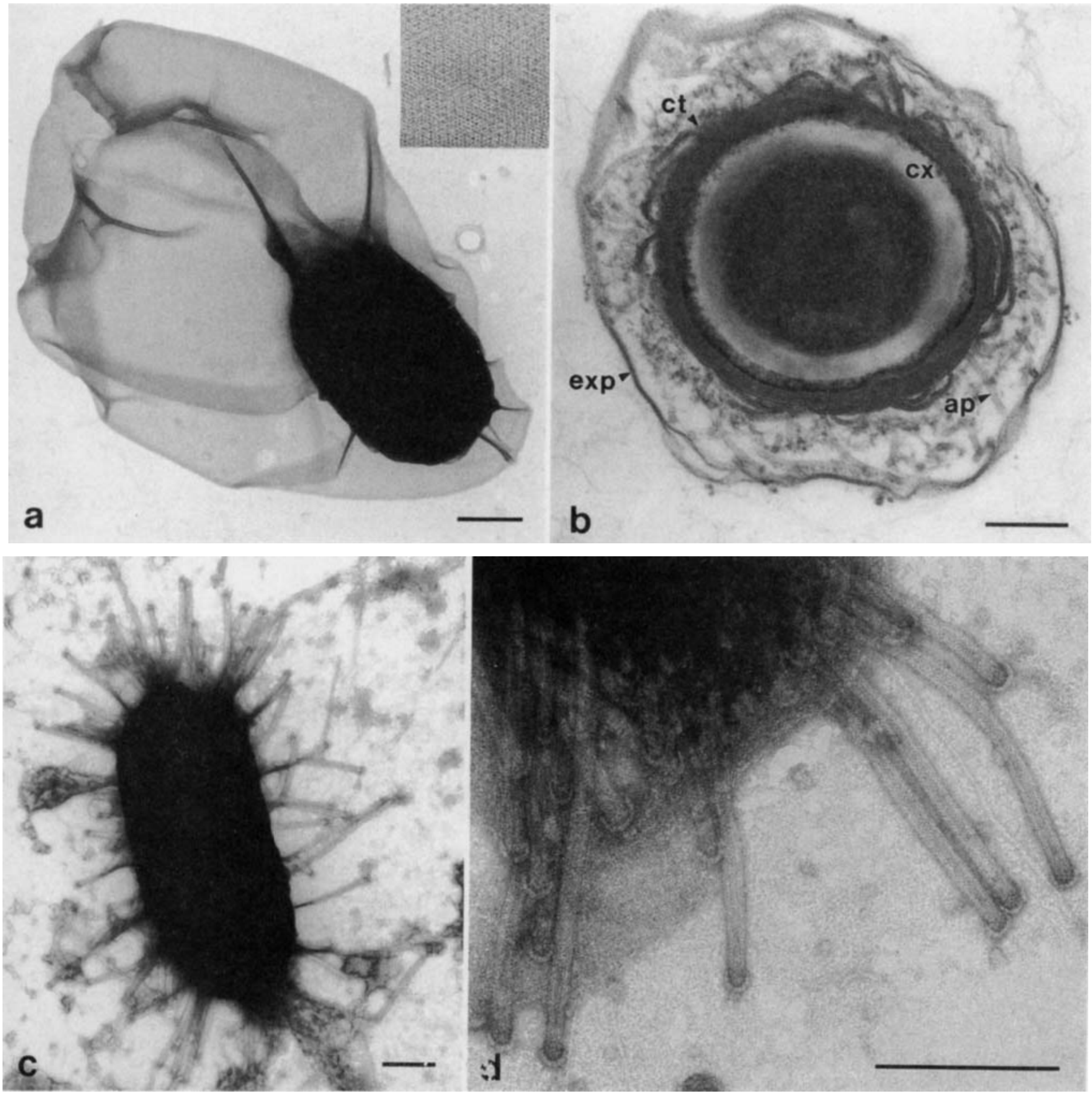

Figure 41–1 Electron Micrographs of Spores of Nonproteolytic *C. botulinum*. **A.** Spore of strain Eklund 17B, type B, negatively stained with saturated, aqueous uranyl acetate; showing membranous exosporium. Inset shows detail of the exosporium (× 150,000.) **B.** Section through spore of strain Foster B96, type E, ruthenium red, 0.075%, included in the fixatives and section stained sequentially with uranyl acetate and lead citrate; showing appendages (ap) between the spore coat (ct) and exosporium (exp), cx is the cortex. **C.** Spore of strain Sebald P34, type E, after removal of exosporium negatively stained as (A), showing appendages. **D.** Spore of strain Hazen 36208, type E, negatively stained as (A), showing detail of appendages. All scale bars represent 200 nm. *Source:* Micrographs courtesy of Dr. M.L. Parker and Dr. M.W. Peck, Institute of Food Research, Norwich, UK.

asthenia gravis, as well as with the effects of other food-poisoning bacteria.

In the early 1900s, 71% of cases of botulism in the US were fatal, but by the 1970s this had been reduced to 15%.[125] The greatest factor in this improvement is said to be improvement in supportive care. A high proportion of patients with foodborne botulism need respiratory support. In the US, treatment by intravenous infusion with therapeutic, trivalent botulism antitoxin is recommended immediately after diagnosis is made, without waiting for laboratory confirmation. By the time clinical symptoms of botulism are present, toxin has bound irreversibly, blocking release of acetylcholine, and antitoxin will only neutralize circulating toxin that is not bound irreversibly to receptors. The antitoxin is prepared from horse serum and its use involves the risk of hypersensitivity reactions. In 268 patients treated with antitoxin in the US between 1967 and 1977, 9% suffered adverse reactions, 2.6% developed urticaria, 1.9% developed anaphylaxis, and 3.7% developed serum sickness.[125] According to some reports, guanidine given orally may be useful in the treatment of botulism;[265] it increases the endplate potential by increasing the amount of acetylcholine released at nerve endings.[292]

Infant botulism was described first in California in 1976 but cases have been reported since in many countries. The majority of reported cases have been caused by bacteria producing type A or B toxin, but organisms producing types E and F toxin have occasionally been implicated; a single report implicated type C toxin. Infant botulism usually occurs in infants less than 35 weeks old and is caused by ingestion of spores of the causative organism; there is evidence that between 10 and 100 spores can cause the infection.[206,292] Numbers of *C. botulinum* as high as 6.0×10^8 colony forming units (cfu) g^{-1} have been reported in the feces of infants with infant botulism.[345] The disease is characterized by constipation, generalized weakness, and other neurological symptoms.[206] This illness is restricted to young infants probably because the intestinal microflora in these infants is insufficiently established to prevent colonization by *C. botulinum* and related organisms. Healthy infant mice are susceptible to colonization by *C. botulinum* spores for a very limited period of their life. Adult germ-free mice and adult mice treated with antibiotics are susceptible also, but become resistant when the mice acquire intestinal microorganisms that are present in normal, adult mice.[32]

In treating cases of infant botulism, equine antitoxin is avoided because of the risk of adverse reactions and of sensitizing the child at such an early age. The use of antibiotics is also not recommended because of the risk that the resulting lysis of the bacteria may result in an increased release of botulinum toxin in the intestine. To test an alternative treatment for infant botulism, a randomized, placebo-controlled trial of human botulism immune globulin (BIG) was started in California in 1992.[12] This study used antibody preparations from the serum of immunized human volunteers (immunized because they were involved in laboratory work on *C. botulinum*). Results from this study showed that intravenous treatment with human BIG was a safe and effective treatment for infant botulism due to proteolytic *C. botulinum* types A and B. For example, the mean hospital stay was reduced from approximately 5.5 weeks to approximately 2.5 weeks, and the mean hospital cost was reduced from $127,900 to $59,200.[13]

The few reported cases in which *C. botulinum* appears to have become established as an intestinal infection in adults[50,197,292] may have occurred when competing bacteria in the normal intestinal microflora have been suppressed by treatment with antibiotics.

According to Smith and Sugiyama,[292] wound botulism is caused only by Group I strains of *C. botulinum*, and the period between infection of the wound and development of symptoms tends to be relatively long, between 4 and 14 days. This incubation period presumably depends on the time needed for conditions in the wound to become suitable to allow growth of the organism and formation and spread of the toxin.

41.4 MECHANISM OF PATHOGENICITY

41.4.1 Structure of Neurotoxins

The major factors responsible for pathogenicity of *C. botulinum* are the toxins (BoNTs). These toxins are neurotoxins, with the exception of C_2 which is of a different type and is cytotoxic.[214,274,292] BoNTs are formed initially as single-chain polypeptides with a molecular weight of about 150 kDa, corresponding to between 1,251 and 1,297 amino acids,[249] and relatively low toxicity[62] and are thought to be released by cell lysis. The single-chain form (protoxin) is converted to a di-chain form (a process termed "nicking"[62]) by extracellular bacterial protease or by an added protease, such as trypsin, to form a molecule consisting of a light chain (L chain ~ 50 kDa) and a heavy chain (H chain, ~100 kDa) linked by a disulphide bond. The bacterial protease and trypsin may attack the same bond or closely associated bonds.[62] Nicking is accompanied usually by activation of the toxin, giving an increase in toxicity of up to 500-fold. Nicking appears to be essential but not sufficient for full activation of toxin[62,286] and there is evidence that conformational changes may be involved also in activation.[62,128] In cultures and in foods, the BoNT may be present either as the single-chain (un-nicked) or as the di-chain form, or as a mixture of the two, depending on the physiology of the organism, the time of incubation,[62] and conditions in the culture or food. The disulphide bridge can be severed by treatment with dithiothreitol, resulting in two nontoxic components. Growth of strains of Group I, proteolytic *C. botulinum* is accompanied usually by the production of proteases that activate the protoxin to highly active toxin, but in the case of Group II, nonproteolytic organisms, the toxin is formed mainly in the poorly active, single-chain form, and in laboratory tests trypsin must be added to activate the toxin in order to demonstrate the potential toxicity.

Toxins of the same type formed by different strains of *C. botulinum* are not necessarily identical.[124] This is shown by the fact that their reactions with a single antitoxin may differ quantitatively (Section 41.8.3). Studies of the nucleotide sequences of representative strains of each of the seven toxin types have also shown differences between the toxin genes from strains producing toxin of the same antigenic type, indicating differences between the amino acid sequences of toxins of the same type from different strains.[54,211]

BoNTs occur in complexes of four molecular sizes, in three of which the toxin is associated with nontoxic proteins (Table 41–3).[128] The M and L forms, referred to as progenitor toxins, are the forms usually found in foods. Progenitor toxins are, in general, more stable at high temperatures and low pH than purified derivative toxin, and their greater stability may prevent or reduce breakdown of toxin in the stomach. RNA is associated also with some toxin complexes but has no known role in activity or stability.[271]

The nature of the food or the culture medium in which growth of the bacterium occurs and the presence of metal ions can affect the size of the toxin complex formed and hence the stability of the toxin. During incubation in foods at 30° C for 7 days, type A strain 62A formed the more stable 16S (L) and occasionally 19S (LL) toxin in string beans and 16S complex in mushrooms, whereas mainly 12S (M) toxin was formed in tuna occasionally with 16S toxin in pork.[311] The proteolytic type B strain Okra formed the 16S complex in all these foods but also produced the less stable 12S complex in tuna and in pork. Type E strain German sprats was tested in mushrooms and tuna, where it only formed 12S complex, and the proteolytic type F strain Langeland formed only the 12S complex in these four foods. Uncomplexed, derivative toxin was not detected. The addition of 5 mM $FeSO_4$ or 5mM $MnCl_2$ to string beans resulted in formation of the 12S rather than the 16S complex by the type A strain, and the addition of 10 mM $FeSO_4$ or 10 mM $FeCl_2$ resulted in formation of the 12S complex rather than the 16S complex by the type B strain.

At pH values below 6.5, the toxin complexes appear to be stable, but if the pH is raised above 7.5 the complexes dissociate, liberating the free neurotoxin. This phenomenon is used in purification of the BoNT.[128,283] The dissociated complexes re-form spontaneously if the pH is lowered. The neurotoxin can be separated from nontoxic components by ion exchange chromatography.

Studies of the neurotoxin genes and the amino acid sequences of toxins of strains representing each toxin type have shown that the L chain is composed of about 450 amino acid residues and contains a zinc-binding site;[275] in representative strains of types A, B, and F, there is evidence that one atom of zinc is bound to the L chain. The crystal structure of type A botulinum neurotoxin has been reported recently.[174]

41.4.2 Control of Neurotoxin Formation

There is some uncertainty about the location of genes controlling BoNT production in Group I and II *C. botulinum*. Because it has not been possible to demonstrate the involvement of either a phage or a plasmid, it has been assumed that these genes are located on the chromosome, but there is a possibility that in some Group I strains the toxin gene is part of a prophage genome present in the chromosome.[211] About 50% of type A strains that formed BoNT/A contained an unexpressed gene for BoNT/B production.[57,96] The genes for BoNTs C_1 and D are present in pseudolysogenic phages, whereas the gene for production of C_2 toxin appears to be located on the chromosome. Nontoxigenic derivatives of type C and type D strains of *C. botulinum* were indistinguishable from each other and from nontoxigenic strains of *C. novyi* type A, and a strain of nontoxigenic *C. botulinum* type C could be converted back either to toxigenic *C. botulinum* type C or type D or to *C. novyi* type A by infection with a bacteriophage carrying the gene for the appropriate toxin.[78] The gene for BoNT/G is reported to be on an 81-MDa plasmid.[355] The location of the toxin genes in toxinogenic strains of *C. butyricum* and *C. baratii*

Table 41–3 *C. botulinum* Neurotoxins and Toxin Complexes

Complex	*Approximate Mass*	*Components*	*Toxin Types*
S "derivative toxin"	150 kDa (7S)[a]	Single chain (heavy{H} chain, ~100 kDa linked to light{L} chain, ~ 50 kDa)	
M "progenitor toxin"	300 kDa (12S)	**S** + nontoxic nonhemagglutinin (NTNH)	A,B,C,D,E,F
L "progenitor toxin"	450–500 kDa (16S)	**M** + hemagglutinin (HA)	A,B,C,D,G
LL	900 kDa (19S)	Probably a dimer of **L**	A

[a] Sedimentation coefficient; S = Svedberg unit

Source: Data from N.P. Minton, Molecular Genetics of *Clostridial* Neurotoxins, in *Clostridial Neurotoxins*, C. Montecucco, ed., pp. 161–194, © 1995, Springer, Berlin, and G. Sakaguchi, *Clostridium Botulinum* Toxins, *Pharmacology and Therapeutics*, Vol. 19, pp. 165–194, © 1983, Elsevier Applied Science.

has not yet been shown definitively.[211] During repeated culture in the laboratory, some strains of *C. botulinum* gradually tend to lose the ability to form high concentrations of toxin and good production of toxin tends to be associated with poor sporulation.[271]

41.4.3 Mechanism of Action of Neurotoxins

The BoNTs act primarily at peripheral cholinergic synapses blocking release of the neurotransmitter acetylcholine; adrenergic systems are affected also, but the dose required to inhibit the release of noradrenaline is much higher than that needed to inhibit release of acetylcholine.[128] Four steps in the action of the BoNTs have been recognized:[274]

1. binding of the heavy (H) chain of the toxin to a specific receptor on the surface of the presynaptic membrane of the motor neuron
2. internalization of the toxin-receptor complex by endocytosis resulting in the formation of endocytic vesicles
3. acidification of the lumen of the endocytic vesicle by a proton-pumping ATPase leading to a conformational change in the toxin, which then inserts into the lipid bilayer of the vesicle membrane; the light (L) chain is then translocated across the membrane of the endocytic vesicle
4. the L chain is freed in the cytosol of the motor neuron by reduction of the interchain disulphide bond, and by means of its zinc-endopeptidase activity, it cleaves protein components of the neurotransmitter-containing synaptic vesicle docking/fusion complex.

Cleavage of one of the proteins within this docking/fusion complex prevents binding of the acetylcholine-containing synaptic vesicles and consequently prevents the release of neurotransmitter and leads to flaccid paralysis of muscle. Each type of neurotoxin cleaves a specific protein involved in neurocytosis. BoNT/B, BoNT/D, BoNT/F, and BoNT/G attack VAMP (synaptobrevin), a protein located on the membrane of the neurotransmitter-containing synaptic vesicle, whereas BoNT/A, BoNT/C, and BoNT/E cleave proteins of the presynaptic membrane, BoNT/A and BoNT/E cleaving SNAP-25 (synaptosomal-associated protein of 25 kDa) and BoNT/C cleaving syntaxin.[274] Each of these toxins is reported to cleave a different, specific peptide bond.[224,274] BoNT/C may also cleave the carboxyl terminus of SNAP-25.[237]

Unlike the other botulinum toxins, toxin C_2 does not have a covalent bond between the L and H chains. It is not neurotoxic but has a cytotoxic effect, which appears to result from its ability to ribosylate actin and disorganize the microfilament network in cells.[128]

41.4.4 Lethality of Neurotoxins

According to Smith and Sugiyama,[292] when assayed by parenteral administration (intraperitoneal, (i.p.) or intravenous, (i.v.) injection), the specific toxicities of the purified, fully activated neurotoxins (S toxins) of the several toxin types range from about 1×10^7 to just over 1×10^8 mouse LD_{50} mg^{-1} protein, equivalent to one mouse i.p. LD_{50} per 0.1 to 0.01 ng protein. The specific toxicities of the bimolecular complex (M complex) and the trimolecular complex (L complex) by parenteral injection are about one half and one third of this, respectively. The specific toxicity of high-quality, crystalline type A toxin complex (900 kDa) should be 3×10^7 mouse i.p. LD_{50}

(± 20%) per mg[271] equivalent to one mouse i.p. LD_{50} per 0.033 ng protein.

Oral toxicity is only a fraction of i.p. toxicity and because complexes are much more stable than the purified toxin, the M and L complexes are much more toxic orally than the S toxin. The estimated minimum amount of toxin needed to cause botulism in humans is based on evidence from incidents of foodborne botulism in which the complexing of toxin in the food was not known.[292] In one case it was estimated that death of a 104 kg man resulted from the consumption of an amount of cheese containing about 3,500 mouse minimum lethal doses (i.p.) of type B toxin.[205] In general, data from cases of botulism in humans following the consumption of food containing toxin indicate that symptoms, and occasionally death, may be caused by as little as 3,000 mouse i.p. LD_{50};[271] this is approximately equivalent to 30 to 100 ng of botulinum toxin depending on the size of the toxin complex.

It has been reported that botulism in humans caused by type B toxin is less severe than that caused by type A toxin. From a study of 309 cases of adult botulism in the US between 1975 and 1988, it was concluded that type A botulism was most severe, as measured by the need for intubation to support respiration, while type E was associated with the shortest incubation periods.[348] No differentiation was made between illness caused by type B toxin produced by proteolytic *C. botulinum* and that produced by nonproteolytic *C. botulinum.* In a study of five strains that produced type B toxin and two that produced type A, all of which formed both M and L toxin complexes in culture medium, purified L toxin from proteolytic type B strain Okra showed much higher oral toxicity in mice than L toxin from the remaining type B and type A strains, whereas the i.p. toxicities were similar.[229] Experiments indicated that the unusually high oral toxicity of the type B toxin from the Okra strain was due to the nontoxic component of the complex and was probably a function of the molecular conformation of the complex.

41.5 INCIDENCE OF *CLOSTRIDIUM BOTULINUM* IN THE ENVIRONMENT AND IN FOODS

41.5.1 Dependence of Results on Methodology

Surveys of the incidence of *C. botulinum* in the environment have been made since the 1920s. Because of the difficulties inherent in detection and isolation of these bacteria, methods used in some of the surveys were subject to various limitations. The conditions optimum for enrichment and isolation of proteolytic, Group I organisms often are not favorable for nonproteolytic, Group II bacteria. For example, the use of heat treatment of samples at 80° C for 10 min to inactivate competing, vegetative bacteria is valuable for isolation from spores of proteolytic *C. botulinum*, but would probably inactivate spores of nonproteolytic strains; treatment of enrichment cultures with ethanol has proved a successful alternative for these.[292] Culture at 35° C is suitable for isolation of proteolytic strains whereas a lower temperature (eg, 26° C) is recommended for isolation of nonproteolytic strains.[299] In many studies, although the type of toxin has been determined, the properties of the organism were not reported, eg, for type B toxin it is not reported whether the bacterium was proteolytic or nonproteolytic.

Growth and isolation of *C. botulinum* can be prevented by competing microorganisms, including *C. perfringens* and *C. sporogenes*, and also by the presence of bacteriophages and bacteriocins (boticins).[165,292] Some samples of sediment from the Fox River in the US were highly inhibitory to Group II *C. botulinum* type E and it was necessary to add 10^6 spores of this bacterium to 1 g of sediment before type E toxin could be detected in culture.[312] The inhibitory effect was due probably to bacteriocins produced by nontoxinogenic Group II *C. botulinum.* Filter-sterilized trypsin can be added to the culture medium to inactivate bacteriocins. Failure to add trypsin to the medium to inactivate bacteriocins or failure to add lysozyme to enable recovery of heat-damaged spores (Section 41.7.3) may lead to failure to detect spores of Group II, nonproteolytic *C. botulinum.* In detecting the presence of toxin produced by Group II, nonproteolytic organisms, treatment of cultures with trypsin to convert the single-chain toxin to the toxic, di-chain form is important for the detection of activity by injection into mice (Section 41.8.2).

In attempts to detect and isolate *C. botulinum* from the environment or foods, it is important, therefore, to assess the adequacy of the method or methods used, by demonstrating that strains of all relevant groups of *C. botulinum* can be isolated following addition of low numbers to test samples from which the organism could not be isolated previously.[312] The detection of *C. botulinum* in a survey will depend also on the size and number of the samples tested. In considering reports of the incidence of *C. botulinum*, therefore, the possible limitations of the methodology used need to be taken into account. In the following discussion it is not practical to review the methodology in each case, but despite this limitation the surveys make it clear that there can be a relatively high incidence of the organism in some environmental samples and foods.

41.5.2 Incidence in the Environment

Quantitative information on the incidence of *C. botulinum* spores in soil and sediment in many parts of the world was summarized by Dodds.[71] The reported incidence of the organism generally is much higher in samples of wet sediments and shoreline samples than in inland soils. In samples of soil and sediment from North America, the percentage of positive samples ranged from 1% in samples of Gulf of Maine sediment to 91% in samples of Washington Bay sediment. The highest estimated number of *C. botulinum* spores was a most probable number (MPN) of 1280 g^{-1} in Green Bay (US) sediment, and in the majority of sediments the organisms isolated most frequently produced type E toxin.[71] In soil samples from the western US, 29% were positive and the majority of isolates (62%) formed type A toxin, whereas in the eastern US, 19% of samples were positive and the majority of isolates (64%) formed type B toxin;[291] while most of the strains producing type B toxin formed the protoxin rather than the active toxin, which usually is considered typical of nonproteolytic *C. botulinum*, according to Hauschild[127] the majority of the North American type B strains are proteolytic.

In surveys in Europe, the reported incidence of *C. botulinum* spores ranged from 0 in samples of Iceland coast sediment and Faroe islands soil to 94% (MPN 2500 kg^{-1}) in samples of sediments in the Netherlands, 98% (MPN 76 kg^{-1}) in samples of sediment from the Norfolk Broads in the UK and 100% in samples of sediment from the Baltic sea (MPN>320 kg^{-1}), the Skagerrak Kattegat sediment (MPN>780 kg^{-1}), and the Sound sediment off the coast of Scandinavia (MPN>1750 kg^{-1}). In the sediments from the Norfolk Broads and from the Netherlands, similar proportions of organisms forming type B, type C/D, and type E toxins were detected, whereas in the other sediments with 100% positive samples, only strains forming type E toxin were detected. Tests using the polymerase chain reaction (PCR) to detect neurotoxin genes indicated that of 110 samples of sediment from the Baltic sea and from the Finnish mainland, 81% of samples of Baltic sea sediment and 61% of samples of freshwater sediment contained *C. botulinum* type E at average MPNs of 940 and 370 spores kg^{-1} respectively.[138]

Surveys in Asia showed that the percentage of positive samples ranged from 0 in samples of sediments from the coast and the Mekong river in Thailand to 75% (MPN 25,000 kg^{-1}) (47% type A, 32% type B, 19% type C/D) in samples of Sinkiang soil in China and 78% (MPN 153 kg^{-1}) in soil from Taiwan (34% type A, 37% type B, 20% type E). A survey for nonproteolytic *C. botulinum* in river soils in Japan showed the presence of type E toxin-producing organisms in up to 82% of samples from some regions and of type B toxin-producing organisms in up to 9% of samples.[350]

In samples from other parts of the world, the reported incidence of *C. botulinum* spores ranged from 0 in samples of soils and sediment from the South Orkney Islands and Brazilian pasture land soil to 34% (mainly type A) in samples of Argentina soil, 35% (mainly type A) in cultivated soil in Brazil, and 55% (all type C/D) in sediment in Auckland, New Zealand.

The isolation of *C. botulinum* from soils is influenced probably by the fact that the estimated numbers of spores of *C. botulinum* in soils are much lower than those of some other *Clostridium* spp. The numbers of *C. pasteurianum*-like bacteria in soils varied between 10^3 and 10^4 g^{-1} dry soil while numbers in the rhizosphere of mangel crops reached 1.4×10^6 g^{-1}.[163] Soils from the Midlands in the UK and from forest and grassland soils in Ghana contained 10^3 to 10^5 N_2-fixing clostridia per g of air-dried soil.[199,200] In six UK Midland soils, counts of 10^4 to 10^6 clostridia spores g^{-1} were found, one of the most common species being *C. perfringens;*[101] and *C. perfringens* type A at levels of 10^3 to 10^4 cfu g^{-1} has been isolated from most of the soil samples examined.[173]

41.5.3 Incidence in Foods

Evidence from Outbreaks of Botulism

Some indication of the widespread occurrence of *C. botulinum* in foods is given by the range of foods that have been implicated in outbreaks of botulism. In addition to fish, meat, and vegetable products, which are the main types of food implicated, and honey, which is associated with infant botulism, unexpected types of food have been implicated, including cheese, hazelnut yogurt, peanuts, and black olives (Section 41.6).

Incidence in Fish

While the incidence of *C. botulinum* in freshly caught fish in many parts of the world can be very low, in fish from some areas the reported incidence can be very high (Table 41–4). The majority of strains detected formed type E toxin. Notably high incidences were reported in fish from the Swedish Sound (100% of samples), Danish trout ponds (between 5% and 100% of samples), Green Bay in North America (56% of samples), and the Southern Baltic sea (95% of samples). On the basis of tests using PCR, of 647 fish of various types from Finnish fishing waters, 15% were positive for organisms producing type E toxin, with Baltic herring showing an exceptionally high prevalence of 40%.[139]

Several studies have shown a high incidence of *C. botulinum* in fish from fish farms. In trout from one Danish trout farm the MPN counts of *C. botulinum* type E were between 340 and 5,300 spores kg^{-1}.[151] This contamination has been associated particularly with farms that were ground-based with earth bottoms.[151] Several measures were shown to contribute to a reduction of this contamination,[152] and in farms with a concrete base[31,35] or that were sea-based with net bottoms,[5] the reported incidence of the organism was much lower. After catching, gutting fish by hand or machine gave a marked reduction in the incidence of *C. botulinum.*[152]

Many surveys for the presence of *C. botulinum* in prepared fish have shown a very low incidence, but in some cases a high incidence and relatively high numbers have been detected (Table 41–5).[72,127] For example, in salted carp from the Caspian Sea *C. botulinum* type E was detected in 63% of samples at an MPN of up to 490 spores kg^{-1}.[267] In dressed rockfish purchased from a wholesaler in California less than 24 hours after landing of fishing boats, all of seven lots were reported to contain *C. botulinum* type A spores at levels of between 90 and 2,400 spores kg^{-1} and one lot also contained 90 type E spores kg^{-1}.[177] Between 30 and 400 spores of *C. botulinum* type A kg^{-1} were detected in a composite muscle tissue homogenate from eight fresh, gutted, dressed king salmon that originated from Alaska, were kept for 3 to 4 days in ice, and were purchased from a wholesale store, and between 5 and 200 spores kg^{-1} were found in a composite sample from nine fresh, migrating salmon caught at a hatchery in California.[98]

Incidence in Meat

There have been relatively few surveys of the incidence of *C. botulinum* in meat (Table 41–6).

In North America, the reported incidence of *C. botulinum* in meat has been relatively low; in an extensive survey, the ratio of nonbotulinal putrefactive anaerobic sporeformers to mesophilic, proteolytic *C. botulinum* spores was estimated as about 20,000 to 1.[114] In two surveys in the UK, *C. botulinum* was not detected in 280 samples of raw pork,[264] but in three further surveys *C. botulinum* was detected in between 2% and 14% of samples in numbers up to 5 spores kg^{-1}; the highest numbers and incidence were associated with a high proportion of type C strains.

In a study of vacuum-packed bacon produced in experimental cures in a commercial curing cellar in the UK, *C. botulinum* types A and B were detected in 4% to 5% of samples from two lots of pigs and type A was detected in 73% of samples from a different lot of pigs[263] (Table 41–6). This appears to be the highest incidence reported in meat products. In these UK surveys, the conditions used for detection would have favored Group I, proteolytic *C. botulinum* as enrichment was at 35° C and the cultures were not trypsinized.

Incidence in Vegetables and Fruits

In a survey in California in the early 1920s, *C. botulinum* types A or B were detected in 15% of 189 samples of fruit and vegetables (Table 41–7).[203] A further survey across the US resulted in the detection of these types in 13% of 431 samples.[204] In several surveys, *C. botulinum* has not been detected in fruit and vegetables, but in some investigations a high incidence has been found. In a survey of modified-atmosphere-packaged vegetables, one package each of shredded cabbage, chopped green pepper, and Italian salad mix were positive for type A and a further salad mix contained type A and type B spores.[176] The survey focused on Group I proteolytic *C. botulinum,* but as these products are intended for refrigerated storage, it is also relevant to consider the incidence of nonproteolytic *C. botulinum,* particularly type B. The high proportion of outbreaks of botulism associated with the consumption of vegetable foods in some countries indicates the potential for the presence of the organism in these foods.

Incidence in Dairy Products

There have been few studies of the incidence of *C. botulinum* in dairy products; only 12 reported outbreaks of botulism between 1912 and 1989 were associated with dairy products, the majority with cheese or cheese spreads,[55] and two further outbreaks were reported in 1996 and 1997.[17,252] The or-

Table 41–4 Reported Incidence of *C. botulinum* in Freshly Caught Fish

Location and Date of Report	*Type of Sample*	*Number of Samples*	*% Samples Positive*	*Toxin Types Detected*[a]	*Reference*
North America					
New York, Lake Cayuga, 1966	Fish	32	6	E(2)	49
Gulf of Maine, 1966	Fish	242	5	E(11)	221
New York to Florida coast, 1967	Fish and invertebrates	382	<1		338
Gulf coast, 1967	Fish	546	4	B(2); C(3); D(1); E(21); F(1)	337
Washington/Oregon coast, 1968	Salmon	369	6	A(2); E(19); F(1)	58
Washington/Oregon coast, 1968	Other fish	170	16	A(1); B(3); E(24)	58
Alaska, 1969	Salmon (entering cannery)	589	1	E(6)	146
Washington, 1969	Salmon (entering cannery)	74	8	E(6)	146
Oregon, 1969	Salmon (entering cannery)	70	6	E(4)	146
Lake Erie, 1970	Fish	363	2	E	312
Lake Superior, 1970	Fish	681	<1	E	312
Lake Huron, 1970	Fish	224	3	E	312
Lake Michigan, 1970	Fish	1,037	8	E	312
Green Bay, 1970	Fish	855	56	E	312
Alaska coast, 1975	Salmon	41	5	E(2)	209
Scandinavia and Europe					
Swedish Sound, 1963	Fish	15	100	E(15)	159
South Baltic, 1963	Fish	11	55	E(6)	159
Kattegat, 1963	Fish	32	0	—	159
North Sea Skagerrak, 1965	Fish	260	0	—	37
Skagerrak, 1966	White fish (whole)	130	0	—	36
Norwegian Sea, 1966	Herring (whole)	29	65	E(19)	36
Viking bank, 1966	Herring (whole)	18	44	E(8)	36
Danish trout ponds, 1974	Trout	550	64	E(357); AE(1); B(1)	151
UK trout ponds, 1975	Trout	1,400	10	B(5); C/D(10); E(88); F(22)	35
Scottish trout ponds, 1975	Trout	69	1	Bnp[b](1)	31
Finnish trout ponds, 1977	Trout	56	7	E(4)	5
Finnish fishing waters, 1996	Burbot, surface	56	18	E	139
	Whitefish, surface	50	18	E	139
	Vendace, whole	50	10	E	139
	Rainbow trout, surface	51	20	E	139
	Baltic herring, whole	53	40	E	139
Asia					
Russia, 1976	Fish and marine animals	~1600	8	A(6); B(13); C/D(4); E(43); F(61)	52
Caspian Sea, 1975	Fish	34	35	E	267
Japan, Hokkaido, 1970	Fish	500	<1	E(2)	171
	Dead fish	12	92	E(11)	171
Japan, Aomori, 1970	Lake trout	110	5	E; F	351
Thailand, 1979	Fish	1 composite[c]	<1	C/D(10); E(5)	319
Indonesia, 1981	Fish	2,103	2.8	A(12); B(10); C/D(32); (F5)	314
Java, marine, 1981	Fish	84	17	A(1); B(3); C(3); D(2); C+D(1); E(2); F(1)	121
Java, fresh water, 1981	Fish	52	13	C(4); D(2); E(1)	121
Central and South America					
Nicaragua/Honduras Caribbean coast, 1967	Fish	82	2	C/D	339
Gulf of Venezuela, Darien, 1966	Fish & invertebrates	28	43	A(5); B(1); C/D(5); F(1)	40
Brazil, 1982	Fish	300	22	A(4); B(3); C/D(3); E(49); F(7)	66

[a] Figure in parentheses is the number of samples in which the toxin type was detected.

[b] Bnp is nonproteolytic *C. botulinum* type B; Bp is proteolytic *C. botulinum* type B; B denotes no differentiation between proteolytic and nonproteolytic.

[c] Composite samples from >16,000 fish.

Source: Adapted with permission from K.L. Dodds, *Clostridium Botulinum* in the Environment, in *Clostridium Botulinum, Ecology and Control in Foods*, A.H.W. Hauschild and K.L. Dodds, eds., pp. 21–51, © 1993, by courtesy of Marcel Dekker, Inc., and A.H.W. Hauschild, *Clostridium Botulinum*, in *Foodborne Bacterial Pathogens*, M.P. Doyle, ed., pp. 112–189, © 1989, by courtesy of Marcel Dekker, Inc.

Table 41–5 Reported Incidence of *C. botulinum* in Prepared Fish Products

Location/Date of Report	*Product*	*Number of Samples*	*% of Samples Positive*	*MPN kg^{-1}*	*Toxin Types Detected*[a]	*Reference*
Fresh Fish						
US, Great Lakes, 1967	White fish chubs eviscerated	78	13	13	E(8); C(1)	234
US, Atlantic coast, 1967	Frozen flounder, vacuum-packed	100	10	70	E(10)	155
England, 1965	General, vacuum-packed	224	0			142
England, 1966	General, vacuum-packed	646	0.8		E(5)	36
Canada, 1967	Frozen, vacuum-packed	400	0.8		E(2); A+B(1)	325
US, New York City, 1985	Filleted cod, whiting, flounder	1,016	0.40		E	250
South Japan, marine, 1980	General	142	7.7	3	C(6); D(1)	120
California, 1986	Dressed rockfish	1 composite	100	90–2400	A; E	177
California, 1990	General fish and seafood	166	21.7	30–440	A; B; E; F	20
California, 1990	Portions of fish fillets	54	67		A; B; E; F	20
Salted fish						
Caspian Sea	General	17	29	170	E(5)	267
Smoked fish						
US, Great Lakes, 1967	White fish chubs	858	1.5	1	E(6); B(1)	234
Denmark, 1967	Salmon	119	1.7	<1	B(2)	222
Baltic Sea, 1967	Eel	10	20	11	E(2)	1
US, Pacific coast, 1970	General	240	19.6[b]	7	E(11)	137
Caspian Sea, 1973	General	64	4.6	24	E(3)	267

[a] Figure in parentheses is the number of samples in which the toxin type was detected.

[b] Toxicity lost in many samples before toxin was typed.

Source: Adapted with permission from K.L. Dodds, *Clostridium Botulinum* in Foods, in *Clostridium Botulinum, Ecology and Control in Foods*, A.H.W. Hauschild and K.L. Dodds, eds., pp. 53–68, © 1993, by courtesy of Marcel Dekker, Inc., and A.H.W. Hauschild, *Clostridium Botulinum*, in *Foodborne Bacterial Pathogens*, M.P. Doyle, ed., pp. 112–198, © 1989, by courtesy of Marcel Dekker, Inc.

Table 41–6 Reported Incidence of *C. botulinum* in Meat and Meat Products

Product and Date of Report	*Number of Samples*	*% Samples positve*	*MPN kg^{-1}*	*Toxin Types Detected*[a]	*Reference*
North America					
Raw pork, beef and chicken, 1966	2,358	0.04		C(1) from chicken	114
Cured meat, vacuum-packed, 1969	NR	NR		B(1)	156
Cured meat, 1967	90	5.5	0.6	B(1)	316
Semi-preserved meat products, 1971	372	1.6	0.5	A(5); B(1)	2
Cured meat, 1980	132	1.5	0.2	A(2)	132
Vacuum-packed bacon, 1980	104	1.0	0.1	A+B(1)	132
Liver sausage, 1980	276	1.8	0.24	A(5)	133
England					
Raw pork, 1977	280	0	<0.1		264
	140	2	0.7	A(2); B(1)	264
	138	14	5	A(3); C(13)	264
	126	5.5	2	C(1)	264
Vacuum-packed bacon, 1977	263	4	2	A(1); B(10)	263
	108 from 34 pigs	5	1	A(3); B(3)	263
	26 from 16 pigs	73	7	A(19)	263

[a] Figure in parentheses is the number of samples in which the toxin type was detected.

NR = not reported.

Source: Adapted with permission from K.L. Dodds, *Clostridium Botulinum* in Foods, in *Clostridium Botulinum, Ecology and Control in Foods*, A.H.W. Hauschild and K.L. Dodds, eds., pp. 53–68, © 1993, by courtesy of Marcel Dekker, Inc., and A.H.W. Hauschild, *Clostridium Botulinum*, in *Foodborne Bacterial Pathogens*, M.P. Doyle, ed., pp. 112–198, © 1989, by courtesy of Marcel Dekker, Inc.

Table 41–7 Reported Incidence of *C.botulinum* in Raw Vegetables and Fruits

Country of Origin and Year of Report	*Product*	*Number of Samples*	*% Samples Positive*	*Toxin Types Detected*[a]	*Reference*
US (California), 1922	Fruit and vegetables	189	19	A(15); B(21)	203
US, 1922	Fruit and vegetables	431	13	A; B	204
Sweden, 1963	Potatoes	40	67	E(27)	159
US, 1969	Chopped spinach	50	12	A; B	156a
US, 1969	Cut green beans	50	0		156a
Former USSR, 1972	Vegetables and herbs	30	43	A; B	268
Canada, 1975	Mushrooms	12	100 (MPN 150–410 kg^{-1})	B	135
Hungary, 1979	Potatoes	26	0		335
Hungary, 1979	Carrots	18	0		335
Italy, 1983	Vegetables	296	4	B	254
US, 1986	Onions	75	6.7	A	297
Germany, 1987	Potatoes, peeled, washed	72	0		22
US (California), 1988	Garlic	115	4.4	A	298
The Netherlands, 1989	Mushrooms	50	0		226
US (Washington, DC), 1990	Cabbages	88	13.6	A	300
US, source companies in Florida, New York, California, 1996	Range of MAP[b] vegetables	1,118	0.36	A(4); B(1)	176

[a] Figure in parentheses is the number of samples in which the toxin type was detected.

[b] Modified atmosphere-packed (MAP).

Source: Adapted with permission from K.L. Dodds, *Clostridium Botulinum* in Foods, in *Clostridium Botulinum, Ecology and Control in Foods*, A.H.W. Hauschild and K.L. Dodds, eds., pp. 53–68, © 1993, by courtesy of Marcel Dekker, Inc., and S.H.W. Notermans, Control in Fruits and Vegetables, in *Clostridium Botulinum, Ecology and Control in Foods* A.H.W. Hauschild and K.L. Dodds, eds., pp. 233–260, © 1993, by courtesy of Marcel Dekker, Inc.

ganism was not detected in any of 40 samples of Edam and Cheddar or 10 samples of cheese spreads.[156] Unpublished studies at the University of Guelph indicated that the number of *C. botulinum* spores in milk was fewer than one per liter.[55]

Incidence in Honey

Following the original recognition of infant botulism in California, a possible correlation was noted between infant botulism cases and feeding of honey. Extensive testing in the US (Table 41–8) showed that some honey samples contained as many as 10^4 spores of *C. botulinum* kg^{-1}.

In few other countries has testing of honey samples been as extensive as that in the US, but the detection of *C. botulinum* in 6.7% of samples of Sicilian honey and in a relatively high percentage of samples tested in Japan shows that the problem occurs more widely than in the US, and *C. botulinum* type B was found in home-canned honey associated with infant botulism caused by a proteolytic, type B strain in Italy.[88] In some of the tests shown in Table 41–8, samples were heated at 80° C before culture; thus spores of Group II bacteria may have been inactivated, but in many cases tests were also made on unheated honey.

Samples of a brand of honey from Argentina that contained up to 6×10^4 spores of *C. botulinum* type F kg^{-1} also contained high numbers of *Bacillus* spp., in particular *B. alvei*.[217] This bacterium is common in bees affected by European foulbrood; it is suspected to cause opportunistic infection in bee larvae, the true cause of European foulbrood being *Melissococcus pluton*, whereas *B. larvae* causes American foulbrood.[218] *B. alvei* enhanced the growth of *C. botulinum* types A and F in dead bees and bee pupae, and it was suggested that contamination of honey with spores of *C. botulinum* results from multiplication of the bacterium in dead bees facilitated by the depletion of oxygen by *Bacillus* spp.[218]

In a survey in the US of other infant foods for *C. botulinum*, 1.3% of 1001 samples of corn syrup contained about 2 *C. botulinum* spores kg^{-1}.[166] Samples of cereals, formula feed, dried milk, whole milk, commercially canned fruit juice, sugar, and fresh cooked carrots were negative. *Clostridium botulinum* spores were not detected in 43 samples of syrups in Canada but were found in 1 of 40 samples of dried cereal;[136] however, these foods were considered to pose a much lower risk than honey.

C. botulinum was detected in 5.4% of samples of sugar for apiculture, 9.1% of samples of raw sugar, 2

Table 41-8 Reported Incidence of *C.botulinum* in Honey[72]

Country and Date of Report	Number of Samples	% Samples Positive	MPN kg^{-1}	Toxin Types Detected[a]	Reference
North America					
US, retail, 1978	55	3.6	80–2.8 x 10^2	A(1); B(1)	313
US, producer, 1978	186	8.6	80–2.8 x 10^2	A(10); B(5); A+B(1)	313
US, 1979	90	10	5 x 10^3– 8 x 10^4	A(2); B(3); B(6)[b]	208
US (product from various countries), 1981	80	7.5		A(4); B(2)	149
US, 1982	100	2		A(2)	166
US, 1983	48	0	<4 x 10^4		115
Canada, 1988	149	0	<2		136
Canada, 1988	1[c]	100	8 x 10^3	A(1)[c]	136
Europe					
Germany, 1980		0	<5		122
Germany, 1980		0	<0.4		95
Italy, 1983	107	0	<0.9		16
Italy, 1983	69	0	<0.5		235
Italy, 1986 (origin Argentina)	2	50		C/D	235
Italy, 1993 (origin Sicily)	30	6.7		B(2)	59
Italy, 1993	1[c]	100		B(1)[c]	88
Norway, 1986		0	<0.3		141
UK, 1987	122	0	<0.4		23
France, 1994	90	0			67
France, 1994 (imported)	26	0			67
Asia					
Japan, 1990 (origin Japan)	58	10		A(3); C(3)	219
Japan, 1990 (origin Argentina)	15	20		A(2); D(1)	219
Japan, 1990 (origin China)	76	11.8		A(3); B(2); C(4)	219
Japan, 1990 (origin Hungary)	12	8.3		A+C(1)	219
Japan, 1990 (origin Mexico)	2	50		C(1)	219
Japan, 1990 (origin Spain)	2	50		A(1)	219
Japan, 1991	36	2.7 (origin Argentina)	10^3–6 x 10^4	F(1)	217
Japan, 1991 (origin Argentina)	13	85	10^3–6 x 10^4	F(11)	217
South America					
Argentina, 1994	42	2.4		A(1)	63

[a] Figure in parentheses is the number of samples in which the toxin type was detected.

[b] The six samples were from honey fed to infants who subsequently developed infant botulism.

[c] The honey was associated with infant botulism.

Source: Adapted with permission from K.L. Dodds, *Clostridium Botulinum* in Foods, in *Clostridium Botulinum, Ecology and Control in Foods*, A.H.W. Hauschild and K.L. Dodds, eds., pp. 53–68, © 1993, by courtesy of Marcel Dekker, Inc.

out of 5 samples of molasses, 2 out of 41 samples of brown lump sugar, 1 out of 8 samples of soft sugar (yellow type), and 1 out of 16 samples of corn syrup in Japan, but not from 27 samples of refined white sugar or 25 other sugar products.[220]

Because of the risk of occurrence of *C. botulinum* spores in honey, warnings have been issued in several countries, including the US, UK, and Italy, not to feed honey to infants less than 1 year old. In the US the Food and Drug Administration (FDA) has advised against the use of honey and corn syrup for feeding infants,[166] and in the UK in 1996, the British Honey Importers and Packers Association advised their members to issue new honey labels with the advice that honey "should not be given to infants under 12 months."[33,190]

Incidence in Other Foods

A few studies have been made of the incidence of *C. botulinum* in a range of prepared foods, particularly vacuum-packed products. In tests of 113 samples, type B was detected in 1 sample of luncheon meat and type E in 1 sample of unshucked oysters.[316] Of 100 samples each of boil-in-the-bag foods, vacuum-packed foods, pressurized foods, and dehydrated and freeze-dried foods, only one sample of vacuum-packed frankfurters was found positive for *C. botulinum*, and it contained type B.[156] In tests for the presence of Group II, nonproteolytic strains in refrigerated, packaged foods on sale in the UK, more than 500 samples were tested including raw trout and mackerel; smoked trout and mackerel; ready meals containing chicken and lasagna; salads containing carrots, lettuce, mixed salad, or watercress; and cooked potatoes either filled or plain.[102] Each sample was mixed with a cooked meat medium (CMM) containing glucose and incubated at 10° C, for three weeks before testing for toxin; none of the samples was positive. Following inoculation of packs of food with approximately 2×10^4 spores of nonproteolytic types B and E, after storage for one week at 10° C, toxin was detected in packs of whole trout but not in smoked trout fillets, lasagna, carrots, or baking potatoes with vegetarian cheese. In these last products either inhibitors, such as an acidic pH, may have prevented growth of *C. botulinum* or the concentration of oxygen and the oxidation-reduction potential in packs may have been too high to allow growth under suboptimal conditions. Except in the case of the whole trout, therefore, the experiments did not allow conclusions to be drawn about the incidence of *C. botulinum* spores in packs, but they indicated that the risk of growth and toxin formation in packs of this type within three weeks at 10° C was very low.

The small proportion of food surveyed gives very limited information about the prevalence of *C. botulinum*. It gives an indication, however, that in some circumstances the bacterium may be present in a high proportion of samples of certain types of food and may give some indication of the factors that lead to the presence of the organism. To ensure the production of safe foods, it must be assumed that *C. botulinum* may be present, but a quantitative assessment of the probable incidence of the organism in foods from a wide range of environments is extremely difficult.

41.6 EPIDEMIOLOGY OF FOODBORNE BOTULISM

In such a severe illness as botulism, it would be expected that the proportion of cases actually reported would be higher than in less severe forms of foodborne disease; nevertheless, cases may be unrecognized or misdiagnosed. Botulism has been misdiagnosed until after death as Guillain-Barré syndrome, chemical poisoning, stroke, or staphylococcal food poisoning.[129] In the US between 1983 and 1996, the main form of botulism reported was infant botulism; in 1996 the number of reported cases of infant botulism (82) was almost four times that of cases of foodborne botulism (22) (Figure 41–2).

The relatively high reported incidence of infant botulism in the US, compared with that in other countries, is probably due in part to awareness of the disease by physicians. The number of cases of wound botulism in the US has increased in recent years and is associated with drug abuse, particularly with the injection of heroin; in 1996 the number of reported cases of wound botulism (17) was only slightly lower than that of foodborne botulism (22).[195]

Reported outbreaks and cases of foodborne botulism in countries that show a relatively high incidence of the disease are shown in Table 41–9, together with the foods and toxin types involved. It should be noted that systems of reporting botulism and criteria for reporting outbreaks and cases differ in different countries.[326] Up until the mid-1980s, the reported incidence was particularly high in Poland and China.

The majority of reported outbreaks of foodborne botulism have been caused by organisms forming type A, B, or E toxin. The first outbreak associated with type F toxin was in the Danish island of Langeland in 1958; four people were affected and the outbreak was attributed to the consumption of home-prepared liver paste contaminated with proteolytic *C. botulinum*.[213] The second occurred in 1966 in California and was due to home-prepared venison jerky contaminated with nonproteolytic *C. botulinum*; of 20 people who ate the jerky, 3 developed botulism, 2 suffered mild gastroenteritis, and 15 remained asymptomatic.[207] Three further incidents were referred to by Hauschild in 1993;[129] type F toxin was the suspected cause of an outbreak in Norway in 1970, type F in combination with type A was detected in an outbreak in Argentina in 1981, and in 1986 type F was associated with a further incident in California.

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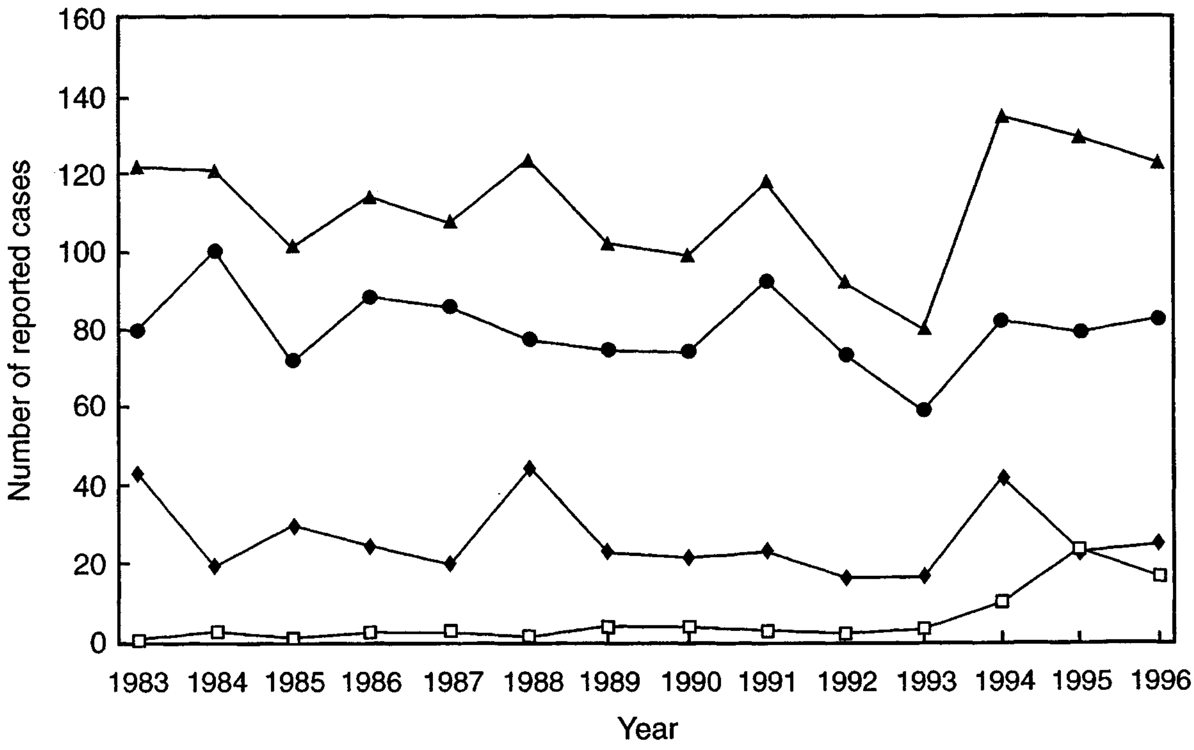

Figure 41–2 Reported Cases of Botulism in the US from 1983 to 1996. ▲, total number of cases; ●, infant botulism; ◆, foodborne botulism; □, wound botulism. Data from reference 195.

A comparison of the number of reported cases of foodborne botulism between 1970 and 1996 in the US, France, Spain, and the UK is shown in Figure 41–3. The number of cases recorded annually in the US has been usually between 10 and 30. In some years a higher number has been reported, several times as a result of restaurant-associated outbreaks. These include an outbreak in 1977 in Michigan, which involved 59 cases and was associated with underprocessing of canned peppers; in 1978 in New Mexico that affected 34 people and was associated with potato salad and/or bean salad; in 1983 in Illinois involving 28 people and associated with temperature abuse of sauteed onions;[129] and in 1994 in Texas that was attributed to consumption of skordalia (a potato-based dip) or meligianoslata (an eggplant-based dip) and affected 30 people.[8] Botulism in France is associated often with consumption of salted, raw hams. Outbreaks of botulism in Spain are attributed generally to consumption of canned vegetables, with asparagus often implicated.[105] In 1987 there was a particularly high reported incidence of botulism in Spain with 11 outbreaks, 46 cases, and 1 death. The foods responsible were determined in six of the outbreaks; five of these were due to home-canned vegetables, the sixth was due to a cured ham.[9] The incidence of foodborne botulism in the UK is low, with a single outbreak reported in each of the years 1978, 1987, and 1989, but the outbreak in 1989 was the largest reported in the UK, affecting 29 people, and was caused by the consumption of a commercially prepared hazelnut yogurt.[233]

Detailed information from countries worldwide was reviewed by Hauschild.[129] The aim of the present discussion is to highlight some of the factors that have led to these cases. The types of food associated with botulism in different countries reflect the methods of food preparation used in those countries (Table 41–9).

Botulism associated with home-preserved vegetables and fruits has resulted (1) when the heat treatment given has been insufficient to inactivate spores of the organism, (2) when growth and acid production by lactic acid bacteria during fermentation has been too slow to prevent germination of *C. botulinum* spores, multiplication of the bacterium, and formation of toxin, or (3) when vegetables pretreated by various means have been covered with oil. The association of botulism with fish products has resulted frequently from insufficient heat treatment

Table 41-9 Reported Foodborne Botulism in Countries with Relatively High Incidence

		Reported Incidence			*Average Incidence*		*Toxin Types Implicated (%)*[a]				*Food Type (%)*[a]				*Food Source (%)*[a]	
Country	*Period*	Outbreaks	Total Cases	Fatal Cases	Outbreaks/ Year	Cases/ Year	A	B	E	Others[b]	Meat	Fish	Fruits/ Vegetables	Others[c]	Home	Commercial
US	1971–1989	272	597	63	14	31	61	21	17	<1	16	17	59	9	92	8
Canada	1971–1989	79	202	28	4	11	4	8	88	0	72[f]	20	8	0	96	4
Argentina	1980–1989	16	36	13	2	4	77	8	0	15	29	21	36	14	79	21
Poland	1984–1987	1,301	1,791	46	325	448	3	94	3	0	83	12	5	0	75	25
Hungary	1985–1989	31	57	1	6	11	0	100	0	0	89	0	4	7	100	0
Yugoslavia[d]	1984–1989	12	51		2	8					100	0	0	0	100	0
East Germany[d]	1984–1989	33	52	4	6	9		>90			52	26	19	3	73	27
West Germany[d]	1983–1988	63	154	6[e]	10	26		>90			78	13	9	0	100	0
France	1978–1989	175	304	7	15	25	0	97	2	<1	89	3	6	2	88	12
Italy	1979–1987		310			34	20	60	7	13	8	8	77	8		
Spain	1969–1988	63	198	11	3	10	0	92	3	6	38	2	60	0	90	10
Portugal	1970–1989	24	80	0	1	4	0	100	0	0	91	9	0	0	100	0
USSR[d]	1958–1964	95	328	95	14	47	33	38	29	0	17	67	16	0	97	3
Iran	1972–1974		314	34[e]		105	0	3	97	0	3	97	0	0		
China	1958–1983	986	4,377	548	38	168	93	5	1	<1	10	0	86	4		
Japan	1951–1987	97	479	110	3	13	2	2	96	0	0	99	1	0	98	2

[a] Where information is known

[b] Includes type F and more than one type of toxin

[c] Includes mixed vehicles

[d] Former countries

[e] Estimate

[f] The meat was mainly that of marine mammals such as seals, whales, and walrus, used by Inuits

Source: Adapted with permission from A.H.W. Hauschild, Epidemiology of Foodborne Botulism, in *Clostridium Botulinum, Ecology and Control in Foods*, A.H.W. Hauschild and K.L. Dodds, eds., pp. 69–104, © 1993, by courtesy of Marcel Dekker, Inc.

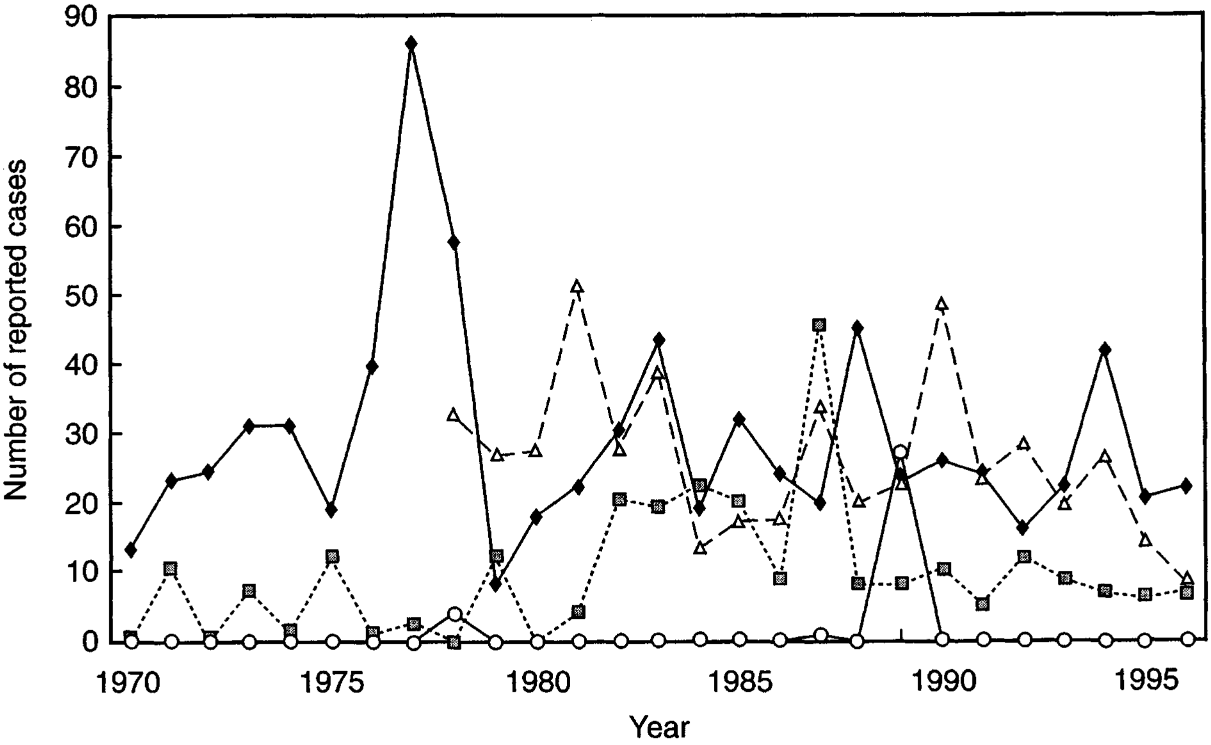

Figure 41–3 Reported Cases of Foodborne Botulism in the US, France, Spain, and the UK from 1970 to 1996. ◆, US; ○, UK; ■, Spain; △, France. Data from references[3,43,64,65,90,191,192,193,195,223,255,334]

or from failure of conditions during fermentation to prevent growth of the organism. In the case of meat products home-preserved by a combination of smoking and addition of sodium chloride (NaCl), botulism has resulted from the failure to achieve a sufficiently high concentration of NaCl to prevent growth of the bacterium.

In Canada, the northern Danish territories, and in many European countries meat products have been the major cause of botulism. A high incidence of botulism was reported from Poland between 1984 and 1987 and was probably due in part to the economic and agricultural situation during this period, which necessitated the home preservation of meats.[179] Between 1984 and 1987, in 830 of 1,791 of the reported cases in Poland, the type of *C. botulinum* involved was determined and 94% of these cases were attributed to type B toxin. The most hazardous practice, which accounted for 60% to 70% of the cases, was reported to be cooking pork in glass jars and storing it under inadequate refrigeration. A relatively large number of outbreaks have been reported from France and Germany, with a high proportion of the cases involving meat products. During World War II, a high proportion of outbreaks in France were due to home- or farm-salted raw hams. The difficult conditions under which pigs were raised and slaughtered, absence of cooling facilities, and shortage of salt contributed to the problem,[179,327] and the high incidence of outbreaks indicates the widespread nature of the bacterium and the potential extent of the problem. In the former West Germany, the number of botulism cases was reported to have declined steadily between about 1960 and 1990, in parallel with a decrease in home canning and curing of foods, particularly meat products.[179] The majority of cases were due to toxic raw hams.

A high proportion of outbreaks in Europe have been due to type B toxin. It has been considered that *C. botulinum* type B strains isolated in Europe were mainly nonproteolytic.[129] Isolates of *C. botulinum* associated with botulism caused by the consumption of raw hams in Central Europe were reported to be nonproteolytic type B strains,[178] but in the case of many of the European outbreaks the causative organisms have not been isolated and characterized. Spores of nonproteolytic *C. botulinum* have generally been considered to have a low heat resistance with a $D_{82.2^\circ C}$ in phosphate buffer pH 7.0 of 0.5 to 2.4 minutes (Section 41.7.3) and on this basis would not be expected to survive the heat processing of the products implicated in the Polish outbreaks. To control foodborne botulism in Europe, there is clearly a need to isolate and characterize bacteria implicated in outbreaks and to determine whether they are Group 1, proteolytic, or Group II nonproteolytic *C. botulinum.*

Fish products have been the major reported cause of botulism in Alaska, Iran, Japan, Norway, Sweden, and the former USSR (Table 41–9).[129] The prevalent isolates in Iran, Japan, and as far as is known in Sweden, produced type E toxin, whereas in Norway strains producing types B and E toxin were each responsible for 47% of cases and one outbreak was associated with type F toxin. Information from the former USSR is limited by the fact that epidemiological work on botulism has been discontinued,[129] but in fish-associated outbreaks between 1958 and 1964, type A toxin was involved in as many outbreaks as type E. The incidence of botulism in native populations of Alaska and parts of northern Canada, in particular in Eskimos and Inuits, is relatively high and is associated with the consumption of fish and marine or land mammals prepared by a process often described as "fermentation" but which, in fact, is usually a putrefaction.[336] Foods such as fish or whalemeat are stored, either raw or partially cooked, often for long periods during which putrefaction occurs because the carbohydrate content of these foods is too low to allow fermentation and sufficient decrease in pH to prevent growth and toxin formation by *C. botulinum.* The major fish products implicated in Iran were salt-cured carp eggs (ashbal); in Japan, fish fermented together with cooked rice, diced vegetables, salt, vinegar, and spices (izushi); and in Norway, raw, fermented trout.

Vegetables have been the major cause of botulism in Argentina, China, Spain, Italy, and the US. With the exception of China, in many of the outbreaks the products were home-preserved by heat treatment, while some outbreaks in Italy have been caused by preservation of vegetables under oil. In China, with a relatively high incidence of botulism, 90% of the reported cases were in the province of Xinjiang (Sinkiang); in outbreaks where the causative food was determined, this was fermented bean curd in 74% of outbreaks and a thick fermented bean sauce in 12% of outbreaks. The predominant causative organisms in Argentina, China, and the US produced type A toxin, whereas in Italy and Spain, type B toxin was involved.

As a result of research over several decades, the controls necessary to prevent the growth of *C. botulinum* in the majority of types of food are well understood. Despite this fact, outbreaks of botulism continue to occur in developed and developing countries. In developed countries, these outbreaks usually are due to failure to understand and apply the known methods of preventing growth of the organism. The majority of outbreaks are attributable to mishandling by home processors or by small-scale processors, but some involve large-scale producers, in which case the consequences of an outbreak can be devastating.

Examples of recent outbreaks that illustrate the continuing problems are shown in Table 41–10.

An outbreak in Taiwan in 1986 was associated with jars of unsalted peanuts in water (ie, a low-acid food) that had been processed by boiling and steaming and was positive for type A botulinum toxin at a dilution of 1:10,000. The producing company was a family-owned business, not licensed to produce canned foods and without a retort device or other steam-pressurized equipment legally required for thermal processing of foods. A recall of the product showed that of 104 jars from the batch implicated, 34 were positive for type A toxin. The authors of the paper stated,

> If other outbreaks of botulism resulted from this or other companies they were either not recognised or not reported. In the current outbreak, about three weeks elapsed from the time of onset of illness among workers in the printing factory until botulism was considered and the public health authorities informed. Clinicians who attended the initial cases assumed the illness was due to chemical exposure in the workplace.

The authors comment that their information suggested that local physicians largely were unaware of the existence of botulism in Taiwan, but that after the publicity resulting from this outbreak, two outbreaks of foodborne botulism associated with home-preserved meats were reported within 4 months and two other sporadic cases were reported.[51] In view of this lack of awareness and underreporting, it is clear that the importance of botulism in Taiwan could not be assessed at least until after 1986.

Following the outbreak in the US and Israel in 1987 due to kapchunka (uneviscerated, salted whitefish), a regulation was introduced to prohibit the processing, distribution, or sale of raw, uneviscerated, salt-cured fish products in New York City.[45,322]

Foodborne botulism in the UK is very rare, but the largest recorded outbreak there occurred in 1989 (Table 41–10) and was caused by the consumption of hazelnut yogurt.[233] Canned hazelnut conserve, an ingredient of the product, was routinely given a mild heat treatment during the canning process, but the

Table 41–10 Examples of Recent Outbreaks of Foodborne Botulism

Outbreak	*Food*	*Number of Cases*	*Number of Deaths*	*Factors Contributing to Outbreak*	*Toxin Type Detected*	*Reference*
1986, Taiwan	Commercially produced jars of heat-processed, unsalted peanuts in water	9	2	Inadequate heat treatment	A	51
1987, Canada	Bottled mushrooms	11	0	Underprocessing and/or inadequate acidification	A	44, 198
1987, US and Israel	Commercially produced, uneviscerated, salted, air-dried fish ("ribbetz" or "Kapchunka")	8	1	Prolonged lack of refrigeration, anaerobic packaging, inadequate salting	E	287
1989, Italy	Black olives	5	0	Faults in preparation. Lack of refrigeration	Bp[a]	89
1989, UK	Commercially produced hazelnut yogurt	27	1	Underprocessing of canned hazelnut conserve	Bp	233
1991, Egypt	Commercially produced traditional, uneviscerated, salted fish ("faseikh")	>91	18	Putrefaction of fish before salting	E	340
1992, United States	Commercially produced "moloha," ethnic, uneviscerated, salt-cured fish product	8	0	Insufficient salt to inhibit *C. botulinum*	E	46
1992, Australia	Home-bottled asparagus preserved in brine	2	0	Insufficient heat treatment and NaCl	NR	236
1992, Spain	Commercially produced green beans/artichokes	4	1	Not known	B[b]	191
1993, Italy	Commercially prepared, roasted eggplant in oil	7	0	Insufficient heat treatment, anaerobiosis	Bp	47
1993, US	Commercially canned cheese sauce	8	1	Contamination of canned cheese sauce after opening (?), growth of *C. botulinum* in the sauce	A	330
1993–1994, Switzerland	Commercially prepared, dry-cured ham	12	0	Not known	B	332
1994, China	Salted, fermented paste made of soybeans and wax gourds	6	NR	Not known	E[c]	202
1994, US	Potato dip "kordalia" and eggplant-based dip "meligianoslata"	30	0	Foil-wrapped, baked potatoes left at ambient temperature for days before use in dip	A	8
1995, Canada (four outbreaks)	"Fermented seal" or walrus meat	9	0	Not reported	E	253
1996, Italy	Commercially prepared mascarpone cheese	8	1	Production process failed to remove or inactivate spores, cheese probably stored above 12° C	A	17
1997, Iran	Traditionally prepared cheese preserved in oil	27	1	Unsafe process	A	252
1997, Germany	Commercially smoked vacuum-packed salmon ("Raucherfisch"), imported	2	0	Suspected temperature abuse	E	158, 172
1997, Germany	Home-smoked vacuum-packed fish ("Lachsforellen")	4	0	Temperature abuse	E	10
1998, UK	Home-preserved mushrooms, imported	2	1	Unsafe process	B	56, 262

[a] Bp, type B toxin produced by a proteolytic strain of *C. botulinum*

[b] B, type B toxin, not reported whether from a proteolytic or nonproteolytic strain of *C. botulinum*

[c] type E toxin produced by a strain of *C. butyricum*. In all other examples, type E toxin was produced by strains of *C. botulinum*

conserve contained a high concentration of sucrose, which would have prevented growth of *C. botulinum* if it had been present. When batches of conserve were prepared in which sucrose was replaced with aspartame, the heat treatment was not increased and growth of *C. botulinum* was no longer inhibited. Growth of the organism occurred in the canned product before it was added to the yogurt. The yogurt was too acid to allow further growth of the organism, but the amount of toxin in the added hazelnut conserve was sufficient to cause a severe outbreak of botulism.

The outbreak in Cairo, Egypt, in 1991 is the largest reported outbreak due to type E toxin and was caused by consumption of a traditional food (faseikh) prepared by salting uneviscerated mullet fish.[340] The fish traditionally were gathered from the Red Sea or from salt-water lakes, but more recently had been obtained from fish farms. They were stored for between several hours and one day to allow swelling and putrefaction before salting and storing them for between 2 weeks and 1 year. As mentioned in Section 41.5.3, fish farming can allow a build-up of *C. botulinum* in sediments and in the fish, and when salt was added during preparation of the faseikh some time would elapse before the salt penetrated through the fish to give a sufficient concentration to prevent growth of *C. botulinum*.

In Italy in 1995 an elderly woman died from botulism suspected to have been due to the consumption of commercially produced, macrobiotic, canned, low-acid food; the heat treatment during processing was at a temperature no higher than 100° C, and was inadequate to inactivate spores of proteolytic, Group I *C. botulinum*.[97] Of 30 samples from the same production lot that were on the market, 10 samples were positive for botulinum toxin (four for BoNT/B and six for both BoNT/A and BoNT/B); a further three samples that were negative for toxin contained spores of *C. botulinum*. Six of the toxigenic strains isolated produced BoNT/B and seven produced both BoNT/A and BoNT/B; none of the isolates produced BoNT/A only.

41.7 FACTORS AFFECTING SURVIVAL, GROWTH, AND TOXIN FORMATION, PARTICULARLY IN FOODS

41.7.1 Differences between Group I Proteolytic and Group II Nonproteolytic *C. botulinum*

The differences between the physiological properties of proteolytic and nonproteolytic *C. botulinum*, outlined in Section 41.2.1, result in different responses to environmental conditions, in cultures and in foods, and to treatments designed to preserve foods (Table 41–11). During growth in foods, proteolytic strains produce off-odors that tend to reduce the risk that the toxic food will be eaten; nonproteolytic strains do not form off-odors to the same extent and toxin is more likely to be formed in the absence of obvious spoilage.

41.7.2 Oxygen and Redox Potential

Spores of *C. botulinum* survive for long periods in air and can germinate in the presence of oxygen. Growth of vegetative cells, however, can be prevented by the presence of oxygen, which increases the redox potential of a food or medium. The complexity of the relationship between oxygen and redox potential is outlined in Chapter 11. In many cases, inhibition of anaerobic bacteria at high redox potential is probably due primarily to the presence of dissolved oxygen rather than to the redox potential itself; several experiments have shown that high redox potential caused by oxidizing agents other than oxygen may not be inhibitory. For example, growth of a butanol-forming clostridium in a culture medium was inhibited by the presence of sufficient oxygen to raise the E_h to +300 mV at pH 7, but in the absence of oxygen the organism grew at an E_h of up to +335 mV when this was caused by the presence of potassium ferricyanide.[170] *Clostridium acetobutylicum* grew at E_h +370 mV at pH 7 when this was maintained by ferricyanide in the absence of oxygen, but was inhibited at E_h +100 mV when this was produced, in the absence of ferricyanide, by aeration, and was inhibited at –50 mV when this was maintained by dithiothreitol in the presence of oxygen.[227]

In mixed cultures or in foods, other microorganisms and/or enzymes can remove oxygen and create reducing conditions that allow growth of clostridia, or foods may be in packs in which oxygen is lowered, in some cases to extremely low levels. The concentration of oxygen in foods may be highly variable and difficult to control or predict; some foods are highly reducing and, in many types of food, it is unwise to rely for safety on the maintenance of a sufficiently high concentration of oxygen to prevent growth of *C. botulinum*.

In experiments to evaluate the effect of other factors on survival and growth of *C. botulinum*, it is essential that conditions for growth should be highly anaerobic so that the results are not influenced by variable, unknown concentrations of oxygen.[180] This

Table 41–11 Factors Affecting Growth and Survival of Group I, Proteolytic and Group II, Nonproteolytic *C. botulinum*

	Group I, Proteolytic C. botulinum	*Group II, Nonproteolytic C. botulinum*
Minimum temperature for growth	10–12° C	3.0° C
Minimum pH for growth	4.6	5.0
Minimum water activity for growth		
(i) NaCl as humectant	0.96	0.97
(ii) glycerol as humectant	0.93	0.94
NaCl concentration preventing growth	10%	5%
Heat resistance of spores in 0.1M phosphate buffer, pH 7	$D_{121° C}$ maximum 0.21 min	$D_{82.2° C}$ in general up to 2.4 min[a]
Radiation resistance of spores at –50° to –10° C[b]	*D* = 2.0–4.5 kGy	*D* = 1.0–2.0 kGy
Toxins formed	A, B, F	B, E, F

[a] Recovery of spores in the absence of lysozyme (in presence of lysozyme, $D_{82.2° C}$ = 231 min)

[b] The preferred temperatures for food irradiation[168]

is illustrated by experiments comparing the probability of growth from spores of *C. botulinum* type E (strain Beluga) at an E_h adjusted to between +62 and +122 mV by the inclusion of a very small quantity of air, and in highly anaerobic conditions at E_h between –426 and –382 mV (Table 41–12).[183] In the presence of 0.1% NaCl, the probability of growth was 10-fold lower at the high E_h than at the low E_h, ie, at the higher E_h growth would be expected from a moderate inoculum, but increased concentrations of NaCl reduced the probability of growth by much greater factors at the high E_h than at the low E_h.

41.7.3 Heat Treatment

Group I, Proteolytic C. botulinum

Early studies of the heat-resistance of bacterial spores were made in the 1920s to develop processes for the production of safe canned foods, following outbreaks of botulism due to inadequately heat-treated products. Experiments involved determination of the thermal death time (TDT) of spores, defined as "the length of time at different temperatures necessary to destroy a definite concentration of spores in a medium of known hydrogen ion concentration.[24] Investigations by Esty and Meyer[84] with 1,804 concentrated spore suspensions of 109 strains of *C. botulinum* suspended in phosphate buffer at pH 7.0 showed that heat treatments at 100° C for 330 minutes, 105° C for 100 minutes, 110° C for 33 minutes, 115° C for 10 minutes, or 120° C for 4 minutes were necessary to prevent growth at 37° C from 6 × 10^{10} spores of three of the most heat-resistant strains. These workers also showed that at 100° C the log number of spores decreased in a roughly linear manner with time over about 10 log cycles and that a very long delay could occur before germination of heated spores. In juices from some canned foods, the heat-resistance of spores was shown to be greater than that in phosphate buffer. Many further experiments were made to determine TDT curves (see Chapter 3) for spores of proteolytic *C. botulinum* and *C. sporogenes* in phosphate buffer and in foods. The use of phosphate buffer (M/15 Na_2HPO_4 and M/15 KH_2PO_4 mixed to give pH 7.0) as a standard heating medium allows comparison of suspensions of spores on a common basis. The concepts of *F* value and *z* value were introduced, where F_0 = the number of minutes required to destroy the organism (in any specified medium) at 250° F (121.1° C) and *z* = the slope of the TDT curve expressed in °F (or later in °C) (ie, the interval in temperature required for the

Table 41–12 The Combined Effect of Redox Potential, Adjusted with Air or by Exclusion of Air and Addition of Reducing Agents, and NaCl on Log Probability of Growth (Log P) from Single Spores of *C. botulinum* Type E at pH 6.8–7.0 and 20° C in 5 Days[183]

	Log P of Growth at the Indicated Redox Potential	
NaCl (%w/w)	E_h –426 to –382 mV	E_h +62 to +122 mV
0.1	0	–1.0
2.5	–	–2.7
3.25	–1.9	–6.1
3.75	–2.9	–
4.0	–4.2	–

Source: Data from B.M. Lund and G.M. Wyatt, The Effect of Redox Potential and Its Interaction with Sodium Chloride Concentration, on the Probability of Growth of *Clostridium Botulinum* Type E from Spore Inocula, Food Microbiology, Vol. 1, pp. 49–65, © 1984, Academic Press Ltd.

line to pass through one log cycle on semi-log paper). Recalculation by Townsend, Esty, and Basalt[331] of the data of Esty and Meyer showed that for the most heat-resistant spores of *C. botulinum* in phosphate buffer $F_0 = 2.45$ minutes and $z = 17.6°$ F (9.8° C).

The decimal reduction time (*D*-value) is defined as the time required for a 10-fold reduction in viable numbers of organisms at a specified temperature. The *D*-value for *C. botulinum* has been determined by two methods:[308] (1) from graphs of log survivors against time and (2) by an assumption of a logarithmic order of death and calculation of a *D*-value from an initial number and a number surviving after a given heating time at each temperature studied (TDT or end-point method). On the basis of many experiments, particularly by the latter method, for spores of proteolytic *C. botulinum* in phosphate buffer pH 7.0, a $D_{121°C}$ of 0.20 minutes and a z value of 10° C (18° F) are used as a measure of the maximum heat resistance and an F_0 value of 3 minutes at 121° C (or a heat treatment of equivalent lethality at other temperatures) has been accepted as a heat treatment that would reduce the number of viable spores of proteolytic *C. botulinum* by a factor of 10^{12} (a 12-*D* process); this has been adopted as the minimum standard for a "botulinum cook" for low-acid foods[308] (see Chapter 3). Several authors (eg, Anderson et al.,[7] Casolari[41]) have questioned whether an $F_0 3$ treatment gives a 12-*D* process for spores of *C. botulinum*. The fact remains that use of this concept has led to the safe production of canned, low-acid foods for many decades, and the majority of cases of botulism due to low-acid canned foods have been due to failure to deliver this process. In practice much greater heat treatments are needed very often to minimize spoilage, because of the presence of spores with greater heat-resistance than those of proteolytic *C. botulinum*. While the number of heat-treated spores that result in growth is affected greatly by components of a culture medium or food, the estimated number of surviving spores of proteolytic *C. botulinum* is not greatly increased by the presence of hen egg-white lysozyme during recovery[6,248,280] in contrast to spores of nonproteolytic *C. botulinum*.

Group II, Nonproteolytic C. botulinum

Spores of these bacteria are much less heat-resistant than those of proteolytic *C. botulinum*. The heat-resistance of spores of nonproteolytic *C. botulinum* is important in relation to foods that are preserved by a combination of mild heat treatment and storage under refrigeration (Section 41.9). Reported *D*-values, determined by TDT methods and by enumeration of survivors, are shown in Table 41–13. The highest *D* values at 82.2° C in phosphate buffer pH 7.0 have generally been in the range of 0.4 to 2.4 minutes.

In fact these heat treatments result in sublethal injury of the spores by inactivation of the germination system.[182,238,241] Lysozyme can enable germination of these injured spores and the addition of lysozyme to the medium used to enumerate survivors results in a marked increase in the number of colonies formed and gives a biphasic survivor curve (Figure 41–4).[6,130,240,281,289] This biphasic curve resulted because lysozyme can diffuse through the coat of only a proportion of the heat-damaged spores (for most strains 0.1% to 1%), but treatment of the heated spores with sodium thioglycollate disrupted the coat of the majority of the sublethally damaged spores; this allowed the ingress of lysozyme and resulted in germination and colony formation, giving a straight line graph of the log number of survivors against time and providing evidence of the mode of action of lysozyme (Figure 41–4).[241] The *D*-values for lysozyme-permeable spores heated in phosphate buffer and recovered in the presence of lysozyme were much greater than those obtained when the spores were recovered in the absence of lysozyme (Table 41–13). Lysozymes from many sources and other enzymes with lysozyme activity, when included in the recovery medium, can increase the number of surviving spores that give colonies after heat treatment.[238,306] Lysozyme activity has been detected in many raw foods, often at a level higher than that required to increase the measured heat-resistance of spores of nonproteolytic *C. botulinum*, and lysozymes can be relatively heat-stable;[182,239] this is potentially important in foods that rely for safety on heat-inactivation of spores of nonproteolytic *C. botulinum*. Studies in which five replicate containers of a model food (meat slurry) per treatment, with no added lysozyme, were each inoculated with 10^6 spores of a mixture of eight strains of nonproteolytic *C. botulinum* showed that while heat treatments at 85° C for 36 minutes or 90° C for 10 minutes were sufficient to prevent growth and toxin formation during subsequent incubation at 25° C for 60 days,[91] when hen egg-white lysozyme (2400 units ml^{-1}) was added to this model food prior to heating, after heat treatments at 85° C for 84 minutes or 90° C for 34 minutes growth was observed at 25° C after 13 and 14 days respectively.[92] In similar experiments a total of 50 containers of meat slurry containing lysozyme (625 units ml^{-1}) each inocu-

Table 41–13 Heat Resistance of Spores of Nonproteolytic *C. botulinum* in Phosphate Buffer and Foodstuffs

Number Strains Tested	*Most Heat Resistant Strain (type)*	*Heating Menstruum*	*Recovery Method*[a]	*Temperature Range (°C)*	$D_{82.2^\circ C}$ *(minutes)*	*z-Value (°C)*	*Reference*
2	Nanaimo (E)	1/15 M phosphate buffer (pH 7)	counts	70–80	1.9	9.6	231
1	Minneapolis (E)	1/15 M phosphate buffer (pH 7)	counts	74–86	1.2	8.3	277
4	Saratoga (E)	1/60 M phosphate buffer (pH 7)	TDT	70–80	0.48	5.6	26
4	Saratoga (E)	1/15 M phosphate buffer (pH 7)	TDT	70–80	0.69	7.2	26
1	1304 (E)	1/15 M phosphate buffer (pH 7)	counts	80	0.52	–	6
3	610 (F)	1/15 M phosphate buffer (pH 7)	TDT	71–85	0.84	6.3	188
8	2129 (B)	1/15 M phosphate buffer (pH 7)	TDT	92–102	32.3	9.7	279
1	17B (B)	1/15 M phosphate buffer (pH 7)	counts	85	0.36	–	240
6	Alaska (E)	1/10 M phosphate buffer (pH 7)	counts	70–90	2.4	10.0	161
5	Alaska (E)	Whitefish chubs	TDT	74–85	2.2	7.6	60
1	Saratoga (E)	Tuna in oil	TDT	72.2–82.2	6.6	6.1	26
1	Saratoga (E)	Sardines in tomato sauce	TDT	72.2–82.2	2.9	6.3	26
5	Beluga (E)	Blue crab meat	TDT	74–85	0.74	8.3	189
1	202 (F)	Crab meat	TDT	76.6–85	1.2	6.4	188
1	Beluga (E)	Oyster homogenate	TDT	50–80	0.82	7.6	30
2	17B (B)	Cod homogenate	counts	75–92	8.7	8.6	100
1	Mixture of strains	Menhaden Surimi	TDT	73.9–82.2	1.2	9.8	259
5	Minnesota (E)	Oyster homogenate	TDT	73.9–82.2	0.43	6.6	48
1	Mixture of strains	Meat slurry	TDT	70–90	18	6.8	91
2	17B (B)	1/15 M phosphate buffer (pH 7)	counts (+LYS)[b]	77.5–90	54	12.4	289
2	17B (B)	1/15 M phosphate buffer (pH 7)	counts (+LYS)[c]	75–95	231	7.6	242

[a] Counts—plate counts; TDT—Thermal death time or end-point method

[b] Egg yolk emulsion included in the plating medium

[c] Hen egg white lysozyme included in the plating medium at 625 units ml^{-1} (10 $\mu g\ ml^{-1}$)

lated with 10^6 spores of a mixture of 8 to 12 strains of nonproteolytic *C. botulinum* were heated at 95° C for 15 minutes; growth and toxin formation occurred in seven of the containers after subsequent incubation at 25° C for less than 60 days.[243] A similarly high heat-resistance has been reported for spores of nonproteolytic *C. botulinum* in crab meat. Experiments in which 30 packs of Dungeness crab meat per treatment were each inoculated with 10^6 spores of a mixture of three strains of nonproteolytic *C. botulinum* type B showed that process times of 50 to 60 minutes at 88.9° C or 90.6° C, 30 to 40 minutes at 92.2° C, or 7 to 17 min at 94.4° C were required to prevent the survival of spores, determined by enrichment at 27° C for up to 150 days.[247] Enzymes with lysozyme activity have been reported in fresh crab at a concentration of about 200 $\mu g\ g^{-1}$.[182] Even when the effect of lysozyme is taken into account, however, at 95° C spores of nonproteolytic *C. botulinum* are of the order of 20-fold more heat-sensitive than those of proteolytic *C. botulinum*. A $D_{95^\circ C}$ value of 4.4 minutes was reported for lysozyme-permeable spores of nonproteolytic *C. botulinum* strain 17B in the presence of lysozyme.[242] In similar tests, spores of 8 strains of proteolytic *C. botulinum* gave $D_{95^\circ C}$ values of 35–92 minutes[244] while extrapolation from a $D_{121^\circ C}$ of 0.2 minutes with $z = 10^\circ C$ would give a $D_{95^\circ C}$ of 80 minutes.

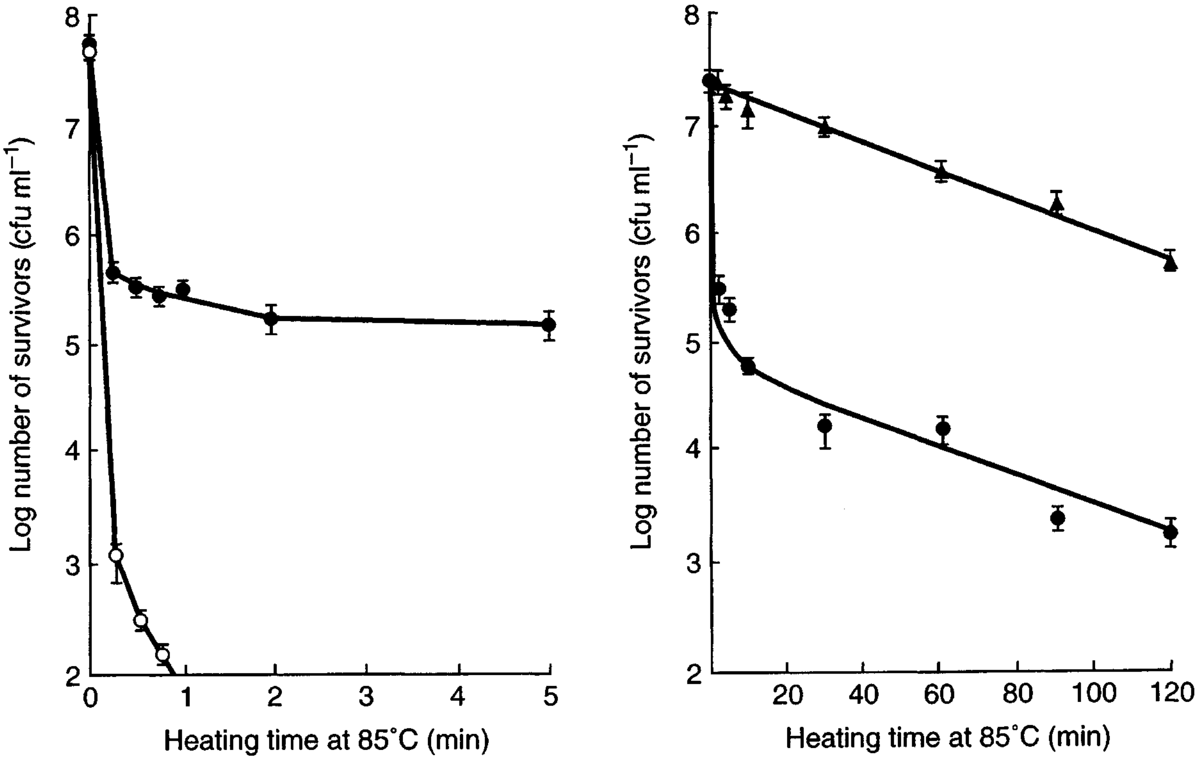

Figure 41–4 The Number of Spores of Nonproteolytic *C. botulinum* Strain 17B That Survived Heating at 85° C in Phosphate Buffer pH7, Estimated by Plating on Medium without Lysozyme (O—O), with Lysozyme, 625 Units (10 µg) per ml (●—●) and after Treatment with Alkaline Thioglycollate before Plating on Medium Containing Lysozyme (▲—▲) and Incubation at 30° C.[240,241] Bars represent 95% confidence limits.

41.7.4 Irradiation

The effect of irradiation on spores of *C. botulinum* has been investigated extensively since the 1960s. With the advice of the World Health Organization that from the point of view of public health there is no upper limit to the dose that can be used,[349] the prospect of using irradiation not only as part of a combination treatment but as a single means of effecting a 12-*D* kill of *C. botulinum* spores becomes theoretically possible. In order to minimize sensory changes in foods, irradiation is done preferably at a temperature in the range of −50° to −10° C.

Survival curves for irradiated spores tend to be log linear against time, often with an initial shoulder.[127] *D*-values for spores of proteolytic *C. botulinum* at −50° to −10° C in neutral buffers and in foods are in the range of 2.0 to 4.5 kGy, while spores of nonproteolytic type E have *D*-values in the range of 1 to 2 kGy.[168] In the temperature range of −200° C to +50° C, *D*-values decrease by approximately 0.01 kGy per °C rise in temperature.

As in the case of heat-treated spores, germination and growth from spores that survive irradiation are influenced by components of the surrounding medium. In addition, growth from irradiated spores is improved markedly by the inclusion of magnesium ions in the culture medium; this may be due in part to the fact that a ligase involved in repair of DNA is dependent on magnesium ions.[168]

41.7.5 Storage Temperature

Group I, Proteolytic C. botulinum

The minimum temperature for growth and toxin production is between 10° and 12° C. Growth and toxin formation at 12° C in 3 to 4 weeks[248,288] and doubling times of 84 to 93 hours at 12.5° C have been reported.[230] Generally no growth has been detected at 10° C or below;[230,248,288] although Tanner and Oglesby[321] reported growth at 10° C from a vegetative inoculum, the work was marred by a temporary rise in the temperature of the incubator to 20° C, and in the words of the authors "the results of this test may not be very significant." These authors did not observe growth at 10° C from a spore inoculum in 10 weeks. In experiments in which 10 g or 20 g quantities of food were mixed with 50 ml of spore suspension containing approximately 6 x 10^8 spores of proteolytic *C. botulinum* and incubated at 10° C, toxin was detected occasionally, and inconsistently, after 4 days or 14 days.[320] This appears to be the only published report of detection of toxin after incubation of food containing proteolytic *C. botulinum* at 10° C. These results may have been due to the release of pre-formed toxin from within the spores, rather than growth and *de novo* synthesis of toxin.[113]

Group II, Nonproteolytic C. botulinum

The minimum temperature at which growth and toxin production have been reported is 3.0° C;[112] in this study, inoculation of 10^5 spores into 10 ml of medium resulted in growth at 3.0° C after 7 weeks. Growth and toxin production had been previously reported at 3.3° C within 31 to 39 days.[79,80,276] Growth and toxin formation have not been detected during incubation at 2.1 to 2.5° C for 90 days.[79,80,112,231,276] Doubling times of a type E strain at 5°, 10°, and 20° C were reported as 42.6 hours, 7.2 hours, and 1.9 hours respectively,[231] whereas for the type B strain 17B doubling times at 5°, 10°, and 20° C were 28.6 hours, 5.7 hours, and 1.9 hours respectively.[109] The fact that there is a risk of growth from small numbers of spores of nonproteolytic *C. botulinum* in foods stored under refrigeration is illustrated in Table 41–14.

41.7.6 pH and Acidulants

The majority of evidence indicates that in otherwise optimal conditions the minimum pH for growth from spores of proteolytic *C. botulinum* is between pH 4.6 and 4.8. Occasional reports of growth and toxin production in high protein media at pH 4.1 to 4.4[290,317,346,354] may be due to the presence of a microenvironment at higher pH. In experiments using vegetative bacteria as inoculum (to avoid any effect of pH on germination, which might be highly variable between spore crops), in a strictly anaerobic medium, adjustment of the pH to 4.6 with hydrochloric acid (HCl) reduced the probability of growth from individual cells of a type A strain at 30° C by a factor of $10^{5.6}$ compared with that at pH 6.8 (ie, provided a safety factor of approximately 6*D*).[185] In the majority of foods, acidity is due to the presence of organic acids such as acetic, lactic, and citric acids, in which case the undissociated acid, or possibly a chelating effect in the case of citric acid,[108] will provide an additional inhibitory effect at an acid pH. Growth and toxin formation by nonproteolytic *C. botulinum* have not been detected at a pH lower than 5.0.[18,111,127] Despite the fact that at 20° C and below growth was prevented at pH 4.5 to 5.1 in the presence of citric acid, spores of proteolytic and nonproteolytic *C. botulinum* survived for 56 days with no significant decrease in numbers.[186]

41.7.7 NaCl and Water Activity

Growth of proteolytic *C. botulinum* is prevented by 10% (w/w) NaCl, giving a water activity of about 0.93. Two strains were able to grow in a culture medium at water activity (a_w) 0.96 but not at a_w 0.95 with NaCl as humectant, and at a_w 0.93 but not at a_w 0.92 with glycerol as humectant.[18]

Nonproteolytic strains of *C. botulinum* do not grow at an NaCl concentration above 5% w/w.[18,111] In strictly anaerobic conditions (E_h7 between –426 and –382 mV), 4.0% (w/v) NaCl reduced the probability of growth at 20° C from spores of the Beluga strain of type E by a factor of about $10^{4.2}$.[183] Growth from spores of the Beluga strain was initiated at a_w 0.97 but not at a_w 0.96 with NaCl as humectant; growth from vegetative cell inocula occurred at a_w 0.98 but not at a_w 0.97 with NaCl as humectant and at 0.95 but not at a_w 0.94 with glycerol as humectant.[18]

41.7.8 Nitrite

This has been used for many years to inhibit *C. botulinum*, primarily proteolytic strains, in cured meat products and in fish products [see Chapter 9; and Section 41.9]. Many studies have been made to evaluate its effect and, because of the concern about nitrosamines, to investigate alternative inhibitors. The effect of nitrite on growth of *C. botulinum* in meat products is influenced considerably by the pH and by components of the product such as available iron and ascorbate or isoascorbate; this fact can limit the value of studies of the effect of nitrite on *C. botulinum* in culture media and in model foods. Nevertheless, such studies have provided useful information on the mechanism of inhibition.

The effect of heat treatment on proteolytic *C. botulinum* is much greater apparently in the presence than in the absence of curing salts (mainly NaCl, 2.5% to 4.0% and $NaNO_2$, up to about 200 mg kg^{-1}). This effect is due mainly not to an increased destruction of spores but to inhibition of growth

Table 41–14 Examples of Reported Time to Toxin Formation in Foods Inoculated with a Low Concentration of Spores of Nonproteolytic *C. botulinum*

		Time (Days) to Toxin Formation at Specified Temperature							
Food	*Inoculum (spores g^{-1})*	*4° C*	*5° C*	*8° C*	*10° C*	*12° C*	*16° C*	*25–30° C*	*Reference*
Turkey	10^2	–[a]	–	8	–	5	2	1	201
Cod	5×10^1	18	–	8	–	6	–	1	250
Salmon	10^2	21	–	6	–	3	–	1	99
Catfish	10^2	46	–	6	–	–	3	–	256
Red snapper	10^2	NT[b]	–	9	–	3	3	1	20; 177
Red snapper	10^3	21	–	9	–	3	3	1	20; 177
Cooked cauliflower	10^3	–	19	17	13	–	4	2	38; 39
Cooked mushroom	10^3	–	20	10	10	–	3	1	38; 39

[a] Tests not carried out at this temperature

[b] No detectable toxin formed in 60 days

from the heated spores by the curing salts.[154] During heat processing and storage of meat products, the concentration of nitrite decreases due to interaction with other components. For nitrite to contribute to inhibition of *C. botulinum*, the starting concentration must be sufficiently high that the residual nitrite level is inhibitory. There is evidence that inhibition of *C. botulinum* by nitrite at concentrations used in cured meats is due to an inactivation by nitric oxide of iron-sulphur proteins such as ferredoxin and pyruvate oxidoreductase within the germinated cells.[329]

41.7.9 Sorbic Acid

Group I strains of *C. botulinum* have been reported as relatively resistant to sorbic acid, which can be used as a carbon source by these bacteria.[352,353] The use of media containing 1,200 mg sorbic acid kg^{-1} was suggested for selective enrichment of *Clostridium* spp. including *C. botulinum*. The only Group II strain tested was less resistant to sorbic acid than Group I strains and did not use a significant quantity of the acid. Despite these reports, there is evidence that potassium sorbate at a concentration up to 2,600 mg kg^{-1} (2,000 mg sorbic acid kg^{-1}) could be used to replace a high proportion of the nitrite added to cured meat products as an inhibitor of *C. botulinum*[296,344] and that addition of potassium sorbate to poultry products,[118,147] ham and bacon,[148] and cheese spread[29] delayed growth and toxin formation by this bacterium.

The maximum concentration of sorbic acid permitted in many foods in the UK and Europe is 1,000 mg kg^{-1} or mg l^{-1}, and in some foods, including processed cheese, is 2,000 mg kg^{-1}.[86,303] In the US, the maximum permitted level of added sorbates as sorbic acid is between 1,000 and 2,000 mg kg^{-1} with some exceptions (eg, in regular cheese the maximum level must not exceed 3,000 mg kg^{-1}).[294,295] A sorbic acid concentration of 2,000 mg l^{-1} had little or no effect on growth of vegetative *C. botulinum* type A strain ZK3 at a pH of 6.0 to 7.0 and 30° C but at pH 5.5, 1,828 mg total sorbic acid l^{-1}, equivalent to 281 mg undissociated sorbic acid l^{-1}, reduced the probability of growth in 14 days by more than 5 logs compared with that at pH 5.5 in the absence of sorbic acid.[184] Similar results were obtained with four further type A strains and five proteolytic type B strains. After growth had occurred, the majority of the sorbic acid was degraded. Germination of spores of proteolytic *C. botulinum* at 35° C and pH 6.5 was inhibited by 12,839 mg sorbic acid l^{-1} (equivalent to 229 mg undissociated sorbic acid l^{-1}), at pH 5.7 by 3,881 mg sorbic l^{-1} (equivalent to 229 mg undissociated sorbic acid l^{-1}) and at pH 5.5 by 1,941 mg sorbic acid l^{-1} (equivalent to 298 mg undissociated sorbic acid l^{-1}).[25,293]

The effect of sorbic acid on growth of nonproteolytic *C. botulinum* has received less attention than that on proteolytic strains. At 20° C and pH 5.5, 1,796 mg sorbic acid l^{-1} (equivalent to 276 mg undissociated sorbic acid l^{-1}) reduced the probability of growth in 60 days from single vegetative cells of nonproteolytic type B strains by >3 logs while 1,300 mg sorbic acid l^{-1} (equivalent to 200 mg undissociated sorbic acid l^{-1}) reduced the probability of growth by only approximately 1 log,[187] whereas at 12° C and pH 5.5, 974 to 1,300 mg sorbic acid l^{-1} (equivalent to 150 to 200 mg undissociated sorbic acid l^{-1}) reduced the probability of growth by about 5 logs.

At pH 5.5 or lower, sorbic acid at concentrations commonly used in foods can provide a considerable safety factor preventing growth of *C. botulinum*, provided that the fat content of the food is not sufficient to take up a high proportion of the undissociated acid; in combination with other factors such as refrigeration, this safety factor can be increased.

41.7.10 Other Antibacterials

Other additives that can contribute to inhibition of *C. botulinum* in foods include nisin, which is used in pasteurized cheese spreads, esters of parahydroxybenzoic acid, phenolic antioxidants, polyphosphates,[168] and CO_2; in addition, ascorbates and certain chelating agents enhance the inhibitory effect of nitrite.

41.7.11 Combinations of Preservative Factors

Preservative factors at a level that singly do not give a high safety factor against the growth of *C. botulinum* can be combined to give a high level of protection; the safety of many foods relies on such combinations of preservative factors (Section 41.9). For example, in the absence of a heat treatment the combined effect of pH, adjusted with HCl, and water activity adjusted with NaCl on growth from spores of single strains of type A, proteolytic type B, and type E is shown in Table 41–15. On the basis of studies of the effect of combinations of factors in culture media and in foods, predictive models have been developed of the combined effect of factors such as temperature, pH, and NaCl on growth of proteolytic *C. botulinum*.[19,246,341] In relation to foods that do not receive a heat treatment, the combined effect of pH (adjusted with HCl) and NaCl on time to growth of nonproteolytic *C. botulinum* at chilled temperatures has been investigated (Figure 41–5).[112] Several predictive models have been developed that describe the effect of controlling factors on growth of nonproteolytic *C. botulinum*.[238]

Because of the interest in foods preserved by a mild heat treatment in combination with refrigeration and other preservative factors, several studies have been done of the combined effect of heat treatment and preservative factors on growth from spores of nonproteolytic *C. botulinum*. A model was developed of the combined effect of heat treatment at 70° to 90° C and storage temperature at 5° to 25° C on growth from 10^6 spores of a mixture of strains of nonproteolytic *C. botulinum* in otherwise optimal conditions.[91] In these experiments, in which lysozyme was not added, heat treatments at 70° C for 2,545 minutes, 75° C for 464 minutes, 80° C for 70 minutes, 85° C for 23 minutes, and 90° C for 10 minutes each prevented growth at 8° C for at least 42 days. Experiments in which lysozyme 2,400 units ml^{-1} (50 $\mu g\ ml^{-1}$) was added to the medium before heat treatment showed that a greater number of spores were able to germinate and give growth after heat treatments at 85° C and 90° C than in the absence of added lysozyme, and a predictive model was developed describing the combined effects of heat treatment at 70° to 90° C followed by storage at 5° to 25° C on the time to growth with lysozyme present before heating.[92] The combined effects of heat treatment, pH, NaCl concentration, lysozyme addition, and storage temperature on time to growth from 10^6 spores in a model food system have also been described.[110,245]

41.7.12 Factors Affecting the Stability of Neurotoxins in Foods

The primary concern in preventing foodborne botulism is to control food production so that formation of BoNTs is prevented. Detailed information is available on thermal inactivation of these toxins in foods.[285] In general, the rate of inactivation is reported not to be linear, the toxins are most heat-stable at about pH 5.0, and food components can protect the toxins against heat-inactivation. Studies of BoNT/A, BoNT/B, BoNT/E, and BoNT/F in several types of food indicate that a concentration of 10^5 mouse $LD_{50}\ g^{-1}$ was reduced to < 1 $MLD_{50}\ g^{-1}$ by heating at 85° C for 5 minutes or less. BoNTs reportedly are more resistant to irradiation than are spores of Group I *C. botulinum*. Frozen storage at –15°C or lower in several types of food has little or no effect on the activity of BoNTs (in the complexes in which they usually occur in foods), but in some foods the heat-resistance of BoNT/E decreased after frozen storage.[285]

41.8 PRINCIPLES OF DETECTION AND ISOLATION

41.8.1 Cultural Methods for Isolation

Isolation may be required for studies of the incidence of *C. botulinum* in the environment or in foods, for detection of the organisms in foods suspected of being implicated in outbreaks of botulism, or from clinical samples from patients and for investigation of the properties of isolates. The various types of samples present different problems of isola-

Table 41–15 Minimum Water Activity[a] Permitting Growth of Proteolytic *C. botulinum* Types A and B and Nonproteolytic *C. botulinum* Type E from Vegetative Cell Inocula at 30° C and Different pH Values[18]

	pH 7.0		*pH 6.0*		*pH 5.5*		*pH 5.3*		*pH 5.0*
Humectant	Glycerol	NaCl	Glycerol	NaCl	Glycerol	NaCl	Glycerol	NaCl	None
C. botulinum A (ZK3)	0.93[b] *(28.4)*[c]	0.96 *(6.5)*	0.94 *(25.6)*	0.97 *(4.8)*	0.95 *(22.3)*	0.97 *(4.8)*	0.96 *(18.3)*	0.99 *(1.2)*	0.997
C.botulinum B (ATCC 438)	0.93 *(28.4)*	0.96 *(6.5)*	0.94 *(25.6)*	0.97 *(4.8)*	0.95 *(22.3)*	0.97 *(4.8)*	0.95 *(22.3)*	0.98 *(3.0)*	0.997
C.botulinum E (Beluga)	0.95 *(22.3)*	0.98 *(3.0)*	0.95 *(22.3)*	0.98 *(3.0)*	no result	0.99 *(1.2)*	0.97 *(13.3)*	0.98 *(3.0)*	NG

[a] Tests were made at intervals of 0.01 a_w

[b] Water activity

[c] Figure in italics is added glycerol (% w/v) or sodium chloride (% w/v)

NG, no growth

Source: Adapted with permission from A.C. Baird-Parker and B. Freame, Combined Effect of Water Activity, pH and Temperature on the Growth of *Clostidium Botulinum* from Spore and Vegetative Inocula, *Journal of Applied Bacteriology*, Vol. 30, pp. 420–429, © 1967, Blackwell Science, Ltd.

tion. Some of the difficulties in isolation of *C. botulinum* were referred to in Section 41.5.1. As proteolytic *C. botulinum* and nonproteolytic *C. botulinum* are physiologically distinct, different conditions are required for their isolation. Heat treatment of samples is used frequently to eliminate competing vegetative bacteria, heating at 75° to 80° C for 10 to 15 minutes is useful in culturing from spores of proteolytic *C. botulinum*. A lower temperature, eg, 60° C is used sometimes during isolation of nonproteolytic strains, and the addition of lysozyme to the enrichment medium can increase the recovery of the heated spores [Section 41.7.3].[282] Unheated samples should be cultured also to isolate vegetative bacteria or spores that are not fully heat-resistant. Treatment with ethanol is used as an alter-

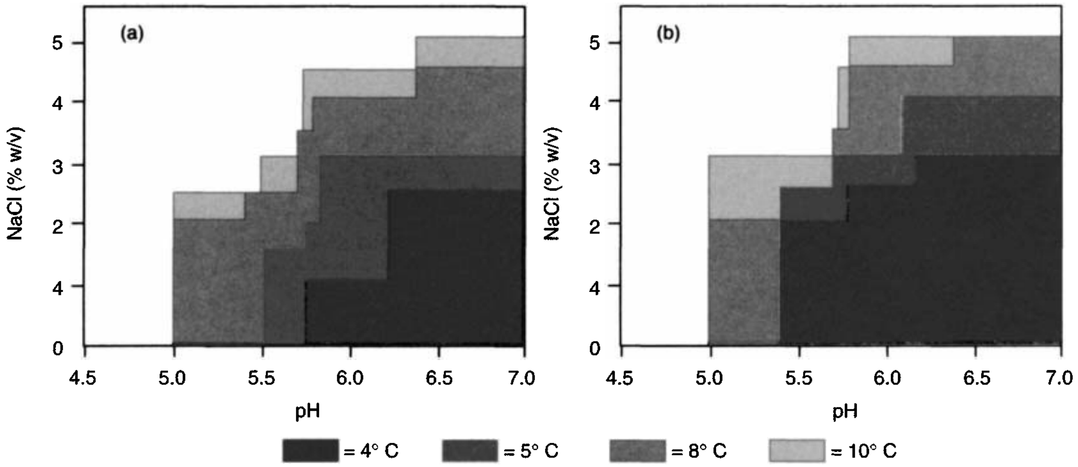

Figure 41–5 Combinations of pH (Adjusted with HCl) and NaCl That Prevented Growth and Toxin Production from Spores of Nonproteolytic *C. botulinum* Types B, E, and F at 4°, 5°, 8°, and 10° C within 3 Weeks (Figure 41–5a) and 6 Weeks (Figure 41–5b). Vials containing 10 ml of PYGS medium were inoculated at 10^4 spores ml^{-1} and incubated at the designated temperature. Intervals of 0.5% NaCl and approx. 0.4 pH units were used. Growth was observed visually and the presence of toxin was confirmed using the mouse test. After 3 weeks, growth was observed at 4° C in vials containing 0.5% NaCl but not in vials containing 0.1% NaCl. *Source:* Data from A.F. Graham, D.R. Mason, F.J. Maxwell and M.W. Peck. Effect of pH and NaCl on growth from spores of non-proteolytic *Clostridium botulinum* at chill temperatures, *Letters in Applied Microbiology*, Vol. 24, pp. 95–100 © 1997, Blackwell Science Ltd.

native to heat treatment for isolation of spores of nonproteolytic *C. botulinum*.[299] Media for enrichment must be anaerobic.[123,167,282,299] In media that are not too highly colored, the redox dye resazurin (which is reduced to the colored resorufin, which is then reversibly reduced to colorless dihydroresorufin, $E'_m = -51$ mV) can be used as an indicator of adequate deoxygenation of the medium[183] (see Chapter 11). To enrich for proteolytic *C. botulinum*, samples should be inoculated into CMM and incubated at 35° C, whereas for nonproteolytic *C. botulinum* CMM glucose medium, chopped meat glucose starch medium[127] or trypticase-peptone-glucose-yeast extract broth containing trypsin (TPGYT) can be used with incubation at 26° C.[299] Trypsin is included to inactivate bacteriocins (boticins) that may be produced by closely related clostridia. Cultures should be incubated for 7 days, or longer if necessary, to allow growth, sporulation, and toxin formation. After enrichment, a portion of the culture can be used to test for BoNT and a further portion plated onto a suitable solid medium. Before plating, the sample of enrichment culture may be treated by heating (in isolation of proteolytic organisms) or with ethanol in order to eliminate competing vegetative bacteria.[299] A suitable nonselective solid medium is egg-yolk agar on which colonies of *C. botulinum* groups I and II have a typical appearance associated with their lipase activity. Several selective plating media have been used including *C. botulinum* Isolation (CBI) agar[69] and Botulinum Selective Medium (BSM);[210] these are useful for detection of proteolytic, Group I *C. botulinum* but the trimethoprin in these media inhibits nonproteolytic Group II strains. Colonies suspected to be *C. botulinum* are characterized by toxin formation and their physiological characteristics.[123,292]

41.8.2 Detection of Neurotoxins by Injection into Mice

Intraperitoneal injection into mice has been the standard method for detection and identification of BoNT,[123] the procedure, controls necessary, and the interpretation are described by Solomon et al.[299] To detect toxin formed by Group II, nonproteolytic *C. botulinum* treatment of samples with trypsin is necessary to convert the single chain toxin to the more toxic dichain form. A collaborative study showed that this method was repeatable and reproducible for the detection of toxin produced by proteolytic and nonproteolytic *C. botulinum* in foods.[164] The specificity of the test is achieved by the use of specific antisera and by observations of typical symptoms of botulism in the mice prior to death. Assays for quantitative determination of BoNT have been developed, and a stable reference standard preparation of BoNT/A was prepared, enabling the response of laboratory mice under a given set of conditions to be assessed and the toxin content of an unknown sample to be expressed in terms of the equivalent of ng BoNT/A g^{-1} or ml^{-1}.[272] Intraperitoneal injection will detect as little as 5 to 10 pg of type A toxin and provides a measure of the biological activity of the toxin.[123] Alternative methods involving a range of animals have been described;[237] these include subcutaneous injection into mice resulting in flaccid paralysis of muscles rather than death, which is as sensitive as intraperitoneal injection but may be less convenient. Advantages of these biological tests are their extreme sensitivity and their ability to detect previously undescribed BoNTs, atypical toxins, and antigenic variants. Disadvantages are the undesirability of using animals and the need to wait several days before a sample can be judged negative.

41.8.3 Detection and Assay of Neurotoxins by ELISA and Other Immunological Techniques

Immunological methods for the detection of BoNTs include enzyme-linked immunosorbent assays (ELISAs) and other tests.[237] Some of the ELISAs have the same sensitivity and specificity as the mouse test and are cheap and easy to use. The ELISA tests have the following limitations:

- some may react with biologically inactive toxin
- some may react differently with toxins of a specific type produced by different strains, since these toxins may differ in antigenicity[74,82,103,104,150]
- some of the tests use antibodies that were raised to preparations containing a mixture of antigens, so that the tests are not specific for BoNTs[150,251,269]
- many ELISAs need a complex and expensive amplification system to achieve the sensitivity of the mouse test.[74,212,284]

Despite these limitations, ELISAs have been used widely. For example, within 2 hours of sample receipt, an ELISA was used to demonstrate the presence of type B toxin in samples of hazelnut purée and yogurt associated with the outbreak of botulism in the UK in 1989.[261] Accounts of at least 20 different ELISAs have been published, some of which are described in Table 41–16.

Immunological techniques have been used also to identify colonies of proteolytic *C. botulinum* types

Table 41–16 Examples of Some of the More Sensitive ELISAs Developed for Detection of *C. botulinum* Neurotoxins

Toxin Type	*Used with Foods*	*Minimum Detection Limit (MLD_{50} ml^{-1})*	*Comments*	*Reference*
A	yes	5–10	No cross reaction with other clostridia, denatured toxin, or other toxin types, but failed to detect toxin produced by one type A strain. Complex amplification system used.	103, 284
B	yes	20	No cross reaction with other clostridia or other toxins, but failed to detect toxin produced by one type B strain. Complex amplification system used.	104, 212
F	yes	10	No cross reaction with other clostridia or toxins of types A, B, or C. Very slight cross reaction with type D toxin; slight reaction with type E toxin.	93
A, B	yes	1–32, <1–16	ELISA may respond to antigens with no neurotoxicity. Correlation between the response from ELISA and mouse bioassay not consistent.	150
A, B, E	yes	9, <1, <1	Cross reaction with some other clostridia. Used extensively to measure toxin production by proteolytic and nonproteolytic *C. botulinum* in meat and in vegetable preparations. Also reacted with strains producing type F toxin.	38, 91, 251
A, B, E	yes	1, 1, 1	Weak reaction with toxin from some strains, suggesting the presence of biologically inactive, but immunochemically reactive, neurotoxin. Used to measure toxin production by nonproteolytic *C. botulinum* in fish fillets. Complex amplification system used (ELCA-ELISA).	74, 75, 266
A	no	1–2	Developed for therapeutic preparations.	82
E	yes	1–10	No cross reaction with other toxins or other clostridia.	347

A and B[106] and nonproteolytic *C. botulinum* type E.[70,106]

41.8.4 Detection and Assay of Neurotoxins by Endopeptidase Activity

Two types of highly specific *in vitro* assay for BoNTs have been developed that are based on their endopeptidase activities. In HPLC-based methods, after incubation of the BoNTs with their substrate (SNAP–25 or VAMP), the substrate and breakdown products are separated by HPLC and assayed by their absorbance.[83,278,342] The HPLC methods are less sensitive than the mouse test and are not suited to rapid detection of toxin in large numbers of samples.

Methods using a combination of endopeptidase activity and immunological reaction have been developed for measurement of BoNT/A and BoNT/B.[81,119] The advantages of these methods over previous ELISA procedures include: (1) they measure the biological activity of the light chain of the toxin (but not of the heavy chain); (2) variations in antigenicity of toxins of a specific type do not influence the response; and (3) the problem resulting from antibodies having been raised to a mixture of antigens is eliminated. In these tests, a fragment of the target protein (SNAP–25 for toxin of type A, VAMP for type B toxin) is attached to a microtiter plate and serves as the substrate. The sample containing neurotoxin is added and, after a period of incubation, specific antibodies are added that bind to the cleaved target protein, followed by secondary antibodies and detection system. Endopeptidase assays have been developed for BoNT/A and BoNT/B that were specific and did not cross react with other BoNTs;[119] the mean detection limits were 260 MLD_{50} ml^{-1} for BoNT/A and 380 MLD_{50} ml^{-1} for BoNT/B. Sensitivity was increased approximately 10-fold by an amplification system. An endopeptidase assay developed for therapeutic preparations of BoNT/A correlated well with the mouse assay and was more sensitive, with a detection limit of 0.2 to 1.0 MLD_{50} ml^{-1}.[81] A combination of endopeptidase and ELISA has many advantages, but further development is required before it can replace the mouse test. Possible problems include interference by other proteases and positive reaction with toxin that is inactive *in vivo*, because the endopeptidase assays relate to the biological activity of the light chain and would not be affected by inactivation of the biological activity of the heavy chain. Assays are being developed that capture the toxin on an immunoaffinity column prior to an endopeptidase assay; these assays show considerable promise in foods and will probably become commercially available.

41.8.5 Use of PCR and Gene Probes To Detect Neurotoxin Genes

Probes have been constructed that enable PCR tests for the nonspecific detection of genes for all types of BoNT[34] and for the specific detection of genes for each of the toxins.[87,94,96,315] Following a cultural enrichment, these methods correlated well with tests for toxin using mice and, with the inclusion of an MPN series of dilutions in the cultural enrichment, have been used for quantitative detection of *C. botulinum* type E inoculated into fish (enabling detection of 210 spores kg^{-1}) and for investigation of the presence of bacteria containing these genes in aquatic sediments and in fish.[11,138,139] The use of a cultural enrichment provides a relatively high number of target bacteria, improves sensitivity, and minimizes possible problems due to the presence of extracellular DNA or to dead bacteria or to interference by food components.

41.8.6 Choice of Method of Detection and Isolation

Cultural enrichments are necessary for isolation and production of pure cultures of organisms that form BoNT and for investigation of their physiological properties. Except in the case of foods in which toxin has been formed, or in some clinical samples, they usually are required prior to tests for toxin.

The use of mice for detection of toxin clearly has been an essential tool in the development of our understanding of *C. botulinum*, in the detection of outbreaks of botulism, and in the understanding of procedures necessary to prevent foodborne botulism. For some time to come, tests in mice may be necessary for reference purposes and for the detection of any neurotoxins of new antigenic types. With the development of the alternative methods described above, however, tests using mice can now be minimized. Increasingly, a great deal of information about the occurrence of these organisms in the environment and in foods can be obtained by cultural enrichment combined with immunological, endopeptidase, or genetic techniques. In studies of factors that affect growth and toxin production by *C. botulinum* in culture media and in foods, which involve tests with known strains, immunological methods can be used very easily for the detection of toxin and, therefore, for confirmation of growth of the organism.[38,91,307] Increasingly, *in vitro* methods can be used to test large numbers of samples for the probable presence of organisms that form BoNT, making possible investigations that in the past have been inhibited by the need for the use of animals.

41.9 CONTROLS TO PREVENT GROWTH OF AND TOXIN FORMATION BY *C. BOTULINUM* IN FOODS

41.9.1 General Principles

Down the centuries, preservation of food has evolved by trial and error until, during the twentieth century, it became possible to evaluate quantitatively the factors that contribute to preservation and safety and to control these factors in order to build in safety. Methods of controlling *C. botulinum* in foods were discussed by Hauschild.[127] The factors that are used to control *C. botulinum* in major categories of food are illustrated by examples summarized in Table 41–17.

The foods that risk being contaminated with the highest levels of *C. botulinum* are probably raw fish from certain sources, and contamination is usually particularly high in the intestine. Evisceration of fish as soon as possible and thorough washing of the fish to reduce the spore load are important steps in reducing the initial level of *C. botulinum.*[76]

41.9.2 Category 1. Shelf-Stable Products, *C. botulinum* Controlled by Heat Processing; Low-Acid Canned Foods

The term low-acid foods is applied in the UK to foods with a pH >4.5[68] and in the US to foods with a pH >4.6.[333] These are considered to be foods with a pH that could allow growth of *C. botulinum*. If canned, these foods must, in general, receive a heat treatment that is sufficient to reduce by a factor of 10^{12} the number of viable spores of proteolytic *C. botulinum* (an $F_0 3$ process). The requirement for thermal destruction of *C. botulinum* in these foods was discussed in Section 41.7.3. In the case of most low-acid, canned foods, heat treatment greater than $F_0 3$ is required to inactivate spores of other species of *Clostridium* and of *Bacillus* with greater heat-resistance than those of *C. botulinum*. While most cases of botulism associated with low-acid canned foods result from underprocessing in the home, some have been caused by commercial products. In recent years, for example, the outbreaks due to heat-processed peanuts, and to hazelnut conserve added to yogurt (Table 41–10), and the case due to a macrobiotic food (Section 41.6) were due to the failure of commercial processors to apply known principles. Following restaurant-associated outbreaks of botulism involving foods heat-processed in-house using faulty techniques, regulations in Canada and in some US states specify that "no in-house (non-commercial) canned or bottled foods may be served in restaurants."[129]

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Table 41–17 Examples of Main Factors Used To Control *C. botulinum* in Foods

Product Category	*Main Factors Controlling C. botulinum*	*Examples of Foods*
Shelf-Stable Foods		
1	Heat process > $F_0 3$	Low-acid, canned foods, pH > 4.5 (UK) or pH >4.6 (US)
2	Heat process < $F_0 3$, NaCl, nitrite	Shelf-stable, canned cured meats
3	Heat process < $F_0 3$, pH	Canned acid foods pH ≤ 4.5 (UK) or pH ≤ 4.6 (US) eg, many types of canned fruit and vegetables
4	a_w (NaCl + drying) ± nitrite. Refrigeration below 5° C during salting	Raw salted and salt-cured meats, eg, salt pork, salt bacon, salt hams, dry-cured hams, and bacon
5	a_w (NaCl + drying) nitrite, pH	Fermented sausages (eg, summer sausage, pepperoni, acidulated sausages)
Perishable Foods		
6	Refrigeration alone	Raw meat, fish, and vegetables
7	NaCl, refrigeration, nitrite in many products, in some cases sorbate, benzoate, nitrate, or smoke	Salted raw fish, lightly preserved fish, semi-preserved fish, hot-smoked fish, perishable cooked, cured meats
8	a_w (NaCl and other components), pH, refrigeration of some products, mild heat treatment	Process cheese and process cheese spreads
9	Mild heat treatment, refrigeration, limited shelf-life	Ready meals, "sous vide" foods

Food poisoning has been caused by post-processing contamination of canned foods. The majority of incidents of this kind have been caused by *Staphylococcus aureus*, but a few have been caused by *C. botulinum*. An outbreak of botulism in the UK in 1978, which affected four people causing the death of two,[21] resulted from the exposure of cans of salmon to contaminated materials during cooling. Measures to prevent such contamination include careful handling of cans, the use of water of good microbiological quality for cooling cans, well-controlled chlorination of cooling water, and regular cleaning and disinfection of conveyers and equipment used for handling cans.[68,140]

There is evidence that some batches of canned foods in which BoNT has been formed have reached the consumer but have not been reported to have caused botulism.[127] For example, in surveys by the US FDA in 1973 to 1974, 20 production lots of commercially canned mushrooms from nine US and foreign canneries contained BoNT but none of the products was involved in botulism. Reasons contributing to this may be:

1. most canned vegetables and some canned meats are heated before consumption, which may inactivate toxin
2. in underprocessed foods, any surviving strains of *C. botulinum* usually would be proteolytic and would produce unpleasant odors in the food, possibly preventing consumption
3. mild symptoms might not be diagnosed and reported.

In general, growth of clostridia in underprocessed canned foods usually leads to gas formation and swollen cans; in canned fish products where the main type of *C. botulinum* present is likely to be nonproteolytic type E, or if canned foods in general are contaminated with a nonproteolytic strain after heat-processing as occurred in the outbreak due to canned salmon noted above, formation of toxin may occur before the food is sufficiently spoiled to prevent consumption. Because spores of putrefactive, nontoxic anaerobes usually outnumber those of *C. botulinum* (by up to 10^4:1) in the environment, the majority of swollen cans are not toxic.[127]

41.9.3 Category 2. Shelf-Stable Products, C. *botulinum* Controlled by Heat-Processing, Sodium Chloride, and Nitrite; Shelf-Stable, Canned Cured Meats

The main products in this category are luncheon meats and canned cured hams. Additional factors that affect the preservation of these products are the pH and iron content of the meat and additives such as ascorbates, isoascorbates, and polyphosphates.[127,134,179] The heat processing given to such products can be between $F_0 0.01$ and $F_0 1.5$,[134] but is usually in the range of $F_0 0.05$ to 0.6. This results in destruction of vegetative microorganisms but not of spores of Group I *C. botulinum*, and the preservation of these foods relies on the combined effect of the heat treatment and inhibition of growth from surviving spores. The magnitude of the protective effect against *C. botulinum* in these foods can be expressed as:

$$\mathrm{Pr} = \mathrm{Ds} + \mathrm{In}$$

where **Pr** = the protection factor (ie, the number of 10-fold reductions in the probability of growth and toxin production per spore)

Ds = the number of 10-fold reductions in viable spores caused by the heat treatment

In = the number of 10-fold reductions in the probability of growth caused by inhibition.[134]

For shelf-stable, canned cured meats with a brine concentration of 5.0% to 5.5%, a nitrite input level of 150 ppm and heat-processed to F_0 of about 0.6, the protection factor was estimated as between 6 and 10, depending on the heat-resistance of the spores used in the experiments.[126] The tendency has been to reduce the brine content of such products, and reducing the level to 4.0% to 4.5% (as used in 1982) would decrease the protection factor by about 2; complete elimination of nitrite from a product containing 5.0% to 5.5% brine would give a similar reduction. Some manufacturers were reported to have compensated for the reduction in salt by increasing the heat treatment of the product, but this increase was limited by adverse sensory effects on the product. Hauschild[126] concluded that data he presented largely supported the view of the Committee of the US National Academy of Sciences/National Research Council that shelf-stable, canned cured meats were likely to become hazardous to health if nitrite was reduced from an input level of 150 ppm or omitted.

From a consideration of the quantities of shelf-stable, canned cured meats that had been produced and sold commercially and had not caused botulism, protection factors varying from >6 to >8.2 were estimated.[134] On this basis combinations of brine concentration, thermal process, and initial nitrite concentration that contributed to this level of safety were summarized (Table 41–18). It was concluded that lowering any one of these factors would require an increase in one or both of the other factors.

41.9.4 Category 3. Shelf-Stable Products, C. *botulinum* Controlled by Mild Heat Treatment and Acidity; Acid Canned Foods

The term acid foods is applied to foods with a pH ≤4.5 (UK) or ≤4.6 (US). The majority of evidence shows that growth of *C. botulinum* is inhibited at pH 4.6 and below, with the possible exception of very rare and specialized conditions. Acid canned foods are given a heat treatment much less than an $F_0 3$ process, but sufficient to inactivate microorganisms that are able to grow at an acid pH. Some botulism outbreaks have occurred due to acid canned foods. Such outbreaks may be due to three possible causes:

1. inadequate heat treatment may allow survival of acid-tolerant microorganisms that subsequently grow, raise the pH, and thus allow growth of *C. botulinum*
2. post-process contamination with acid-tolerant microorganisms may have the same effect
3. the food (eg, vegetables) may have been acidified insufficiently before canning.[127]

All 35 cases of botulism reported in the US in the period 1928 to 1978 and attributed to "acid foods" involved home canning and were associated with prior growth of another microorganism that raised the pH, permitting growth of proteolytic *C. botulinum*.[302]

41.9.5 Category 4. Shelf-Stable Products, C. *botulinum* Controlled by Reduced Water Activity, Sodium Chloride, Sometimes Also Nitrite, and Refrigeration during Salting; Shelf-Stable Raw Salted and Salt-Cured Meats

Examples of products are salt pork, dry-cured bacon, the dry-cured hams produced in continental Europe, and country-style ham produced in the US.[160,328] In raw cured, shelf-stable meats, the main salts used in curing are NaCl and nitrites, the latter being added partly to improve the color and flavor of the product. The cure may be applied as a dry salt, by injecting the meat, or by a combination of these methods. In com-

Table 41–18 Combinations of Brine Concentration and Thermal Process That, in Conjunction with a Nitrite Input of 150 ppm, Were Estimated To Ensure the Safety of Shelf-Stable, Canned Cured Meats from *C. botulinum*, ie, To Give a Protection Factor[a] of at Least 6.6[134]
[% brine = (NaCl x 100)/(NaCl + H_2O)]

Product	*Brine Concentration %*	F_o
Luncheon meats	3.0–4.0	1.0–1.5
	4.0–4.5	1.0
	4.5–5.0	0.5–1.0
	5.0–5.5	0.5
Ham and shoulder	3.3	0.3–0.5
	3.7	0.2–0.3
	4.0	0.1–0.2
Sausages	2.5	1.5

[a] Protection factor calculated as log number of cans produced none of which were reported to have caused botulism

Source: Reprinted with permission from A.H.W. Hauschild and B. Simonsen, Safety Assessment for Shelf-Stable Canned Cured Meats—An Unconventional Approach, *Food Technology*, Vol. 40, No. 4, pp. 155–158, © 1986, Institute of Food Technologists.

mercial production in Europe, brine (containing 10% to 20% (w/v) NaCl and 0.1% to 0.15% (w/v) nitrite or nitrate)[14] is injected usually into the meat. During the initial curing and ripening, the product should be maintained below 5° C to prevent the growth of nonproteolytic *C. botulinum*, particularly type B.[157] In the case of dry-cured hams, when the a_w in all parts of the ham has fallen below 0.96, the temperature of drying is raised first to <15° C then later to 18° to 20° C. The product is smoked at up to 22° C; the a_w of the final ham is less than 0.90. The combination of high content of salt and nitrite and the low a_w resulting from curing and drying makes these products shelf-stable. In Europe home- or farm-produced, dry-salted hams have been a major cause of botulism due to strains forming type B toxin; this occurred particularly during periods of stress such as World War II.[179,327] Factors contributing to these incidents probably included shortage of salt and failure to maintain the hams below 5° C during the early stages of salting.

41.9.6 Category 5. Shelf-Stable Products, *C. botulinum* Controlled by Reduced Water Activity, Acidity, and Nitrite; Dry Fermented Sausages

These products include summer sausage, pepperoni, and acidulated sausages and are described in Chapter 19. The acidity and drop in pH resulting from growth of starter organisms combined with the low water activity of the final product prevent growth of *C. botulinum*, which does not appear to be a hazard in these foods[127] (see Chapter 19).

41.9.7 Category 6. Perishable Products, *C. botulinum* Controlled Mainly by Refrigeration

Examples of food in this category are red meats, which are transported worldwide in vacuum packages in low-permeability plastic film at temperatures of about 0° C; as a result of respiration of the tissue, high concentrations of CO_2 develop. Clostridia are usually not present in deep tissues of meat. They are likely to be present on the surface but on raw meat they tend to compete poorly with other bacteria and spoilage occurs before growth and toxin production by *C. botulinum.*[179] There are, however, reports of spoilage by psychrotrophic *Clostridium* spp. of refrigerated, vacuum-packed raw meat maintained below 3° C.[27,61,162]

The Inuit population of northern Canada and the Eskimo population of Alaska have a history of eating putrid, raw meat of marine mammals. This practice has led to numerous outbreaks of botulism caused by nonproteolytic *C. botulinum*, which is probably favored by the low temperatures. Consumption of raw, fresh meat has not been reported as the cause of botulism in any other part of the world;[179] the main factors that can be used to control safety are probably minimization of contamination of the meat from the intestinal contents at slaughter, maintenance at low temperature, rejection of meat showing spoilage, and cooking before consumption.

Vacuum- and modified atmosphere-packaging are used extensively for commercial, refrigerated bulk shipment of raw fish. The risk of botulism associated with raw fish is greater than that due to raw meats because of the higher rate of contamination with *C. botulinum* spores, and because the predominant strains present are nonproteolytic and therefore toxin formation can occur before noticeable sensory deterioration. In a study in which 100 spores of *C. botulinum* type E 100 g^{-1} sample were inoculated into fresh salmon, which was then stored at 10° C in air, in 60% CO_2 + 25% O_2 + 15% N_2, or 90% CO_2 + 10% N_2, formation of toxin was delayed in the CO_2 atmospheres; but after 10 days, samples in air were spoiled and toxic whereas samples in the CO_2 atmospheres contained toxin but were not overtly spoiled.[76] Thus inhibition of the spoilage microflora by the CO_2 could increase the shelf-life of the fish but increased the risk due to *C. botulinum*. In a further study of salmon fillets surface-inoculated with 100 spores of type E g^{-1} of fish, toxin was detected before spoilage in packs containing 75% CO_2 + 25% N_2 and stored at 16° C but not in packs under air or vacuum or in packs stored at 4° or 8° C.[257] At 4° C sensory spoilage occurred but toxin could not be detected many days later. Experiments of this kind have given different results, probably due in part to differences in the initial quality of the fish, in the initial number of spoilage organisms, in number and properties of the *C. botulinum* strains present, and in the nature of packs. The results indicate, however, that modified-atmosphere packaging (MAP) can delay spoilage and increase the shelf-life of raw fish but does not reduce the risk of growth of *C. botulinum*, and the safety of such products depends on very strict control of temperature to about 4° C or lower. Practices have been used to preserve fresh fishery products in CO_2 in conditions where the temperature can be controlled (eg, at 2° C); the products are then removed for retailing, allowing growth of spoilage bacteria to resume to ensure that spoilage occurs before the product might become toxic.[76]

Outbreaks of botulism have been associated with unexpected foods in which the safety of the product was dependent on refrigeration but temperature abuse occurred. In North America, two outbreaks of botulism have been attributed to a product containing chopped garlic in soybean oil.[216,305] The first of these outbreaks, due to type B toxin, was one of the largest reported in North America, affecting 36 people, while the second, also due to type B toxin, affected 3 people. The commercially manufactured products were labeled "keep refrigerated" (in small print) but were, in fact, kept at room temperature for a prolonged period. The oil content of these products presumably created sufficiently anaerobic conditions to allow growth of *C. botulinum*. After the second of these outbreaks, the FDA ordered companies to stop production of any garlic-in-oil mixes that are protected only by refrigeration, and such products must now contain specified concentrations of antimicrobial agents or of acidulants such as phosphoric or citric acid.[216]

41.9.8 Category 7. Perishable Products, *C. botulinum* Controlled Mainly by Sodium Chloride and Refrigeration, Often in Combination with Nitrite, Sometimes with Other Factors Such As Sorbate, Benzoate, and Nitrate; Salted Fish, Lightly and Semipreserved Fish Products, Hot-Smoked Fish, Perishable, Cooked Cured Meats

An example of a salted, raw fish product is kapchunka, which is prepared by layering raw, uneviscerated fish and salt; after storage for a minimum of 25 days, the fish are rinsed and air-dried at ambient temperature for 3 to 7 days.[76] After drying, the fish should be marketed under refrigeration and often are eaten uncooked. This type of food has caused several outbreaks of botulism (Table 41–10) probably because growth of *C. botulinum* and production of toxin had occurred before an inhibitory concentration of salt had diffused through the fish. The risks associated with raw, salt-cured fish can be reduced considerably by careful evisceration of the fish to reduce the level of possible contamination with spores of *C. botulinum* from the viscera, by ensuring that a high salt concentration is attained as rapidly as possible throughout the fish, and by keeping it under refrigeration during production, distribution, and sale.[76]

Lightly preserved fish include products such as pickled "gravad" fish. These are a Scandinavian food prepared from fillets of salmon, halibut, or trout that are sprinkled with salt, sugar, and spices and are left to ripen at chill temperature for 1 to 2 days (see Chapter 21). Gravad fish often are prepared in the home, and failure to use an adequate amount of salt or to maintain at low temperature has resulted frequently in cases of botulism. Some types of lightly preserved fish also contain sorbate, benzoate, nitrite, or smoke. In vacuum-packed, cold-smoked trout inoculated with a mixture of spores of nonproteolytic *C. botulinum* types B, E, and F to give 810 cfu kg^{-1} or 20,210 cfu kg^{-1}, a water-phase concentration of 3.4% NaCl failed to prevent toxin formation at 4° or 8° C within 6 weeks; toxin was also formed in trout cured with 3.4% NaCl and 166 mg $NaNO_2$ kg^{-1} but not in samples treated with 3.4% NaCl plus 686 mg KNO_3 kg^{-1}.[153] In a further experiment in which vacuum-packed, cold-smoked trout were inoculated with spores of a mixture of strains of type E to give 140 cfu kg^{-1} or 4,140 cfu kg^{-1} a water-phase concentration of 3.4% NaCl failed to prevent toxin formation at 4° or 8° C in 6 weeks but the presence, in addition, of an initial concentration of 109 mg $NaNO_2$ kg^{-1} or 347 mg KNO_3 kg^{-1} prevented toxin formation.

Semipreserved fish products (eg, salted or marinated herring, anchovies, and caviar) usually contain a higher concentration of NaCl than lightly preserved products; some may also contain sorbate, benzoate, or nitrate. Some types of caviar contain >10% NaCl and are shelf-stable, whereas other types contain <10% NaCl and are stored under refrigeration[131] (see Chapter 21). Hauschild and Hilsheimer[131] concluded in 1978 that most of the commercial types of caviar that they examined contained combinations of pH and salt that would inhibit proteolytic

C. botulinum at ambient temperatures. In Europe, many types of caviar are maintained below 10° C, and control of NaCl and refrigeration are important for the safety of these products (see Chapter 21). There are few reports, all prior to 1980, in which caviar has been associated with outbreaks of botulism.[127]

Hot-smoked fish such as salmon, mackerel, and trout are heated commonly at 60° to 80° C.[76] The heat treatment is not sufficient to inactivate spores of nonproteolytic *C. botulinum,* although they may be damaged. The addition of NaCl is of major importance in the control of *C. botulinum,* and both nitrite and smoke can reduce the concentration of NaCl required. After an evaluation of many experiments, Eklund[76] concluded that 3.5% NaCl in the water phase would be adequate to inhibit growth of *C. botulinum* type E in vacuum-packed, smoked fish that are smoked at least during the first half of the process and heated to an internal temperature ≥63° C for a minimum of 30 min. If 100 to 200 mg $NaNO_2$ kg^{-1} was included in the process, the water-phase concentration of NaCl could be reduced to 3.0%, but in the US, $NaNO_2$ was permitted only in a limited number of types of fish.

Perishable cooked, cured meats include emulsified products such as frankfurters and liver sausage, coarse-ground chopped meats, sectioned and formed meats such as ham, prime cuts, and jellied meats. The heat treatment used during production depends on the products, and internal temperatures required by the US Department of Agriculture vary from 60.0° C to 76.6° C.[160,328] Hauschild[126] considered that "these products are safe from *C. botulinum* as long as they are properly refrigerated" and that the risk to be considered was that resulting from temperature abuse. From challenge experiments by several groups of researchers, the probability of toxin production from spores of proteolytic *C. botulinum* inoculated into wieners, chicken frankfurters, turkey roll, and liver sausage during incubation at 27° C for 1 week was determined. In most cases, the probability of a single spore giving rise to toxin in the product in these conditions was $<1 \times 10^{-4}$, ie, the protection factor was greater than 4, but in turkey roll with 3.2% to 3.4% brine and 0 to 50 mg nitrite kg^{-1}, the protection factor was only about 3. In liver sausage with 3.8% to 4.2% brine but with no nitrite, the probability of growth and toxin production in these conditions was about 3×10^{-4}, equivalent to a protection factor of about 3.2, but the addition of 50 to 150 mg nitrite kg^{-1} with the same concentration of brine reduced the probability of growth and toxin formation to $<2 \times 10^{-6}$ in the presence of 150 mg nitrite kg^{-1}, ie, gave a protection factor of about 6.

41.9.9 Category 8. Perishable or Shelf-Stable Products, *C. botulinum* Controlled by Mild Heat Treatment, Reduced Water Activity, Acidity, and Refrigeration of Some Products; Process Cheese and Process Cheese Spreads

During the production of these spreads, cheese mixed with the other ingredients is heated to between 80° and 98° C before filling into the final containers. In the US, pasteurized, process cheese spreads with a pH generally in the range 5.4 to 6.0 and a moisture content of about 50% are mostly distributed as shelf-stable products in hermetically sealed containers.[301,318] In some other countries, these types of products are intended for refrigerated storage, but they may be subjected to temperature abuse. The heat treatment may be sufficient to give a high level of inactivation of spores of nonproteolytic *C. botulinum* but will not inactivate spores of proteolytic strains, inhibition of which will depend on a combination of pH and water activity, controlled by NaCl and other ingredients, and in some cases by refrigerated storage.

Combinations of pH, NaCl plus disodium phosphate, and moisture that prevented toxin formation in process cheese spreads from an inoculum of spores of type A and B proteolytic strains at 30° C have been determined.[318] In further experiments, in process cheese prepared with a_w 0.95 and pH 5.55 and inoculated with spores of a mixture of type A and B proteolytic strains, no growth occurred within three months at 15° to 30° C.[323] In addition to the effects of a_w and pH, the stability of the products was increased by the presence of lactic acid originating from the cheese and decreased by substitution of some of the phosphate emulsifying salt by citrate. The results of a series of experiments, in which the effect of composition of cheese spreads on growth of proteolytic *C. botulinum* was determined, were used to derive a model predicting the effect of composition on time to toxin production.[324] The addition of nisin inhibited growth of proteolytic *C. botulinum* in process cheese spreads; a nisin concentration of 12.5 ppm was effective in some formulations while 100 to 250 ppm was needed to inhibit *C. botulinum* in cheese spreads with the highest moisture levels or with lowest sodium levels.[301]

An outbreak of botulism in Argentina in 1974 caused by a commercial cheese spread with onions focused attention on the safety of these products.[28] A survey of samples of Argentine commercial cheese spread showed that a_w values varied between 0.968 and 0.978 and pH values were between 5.6 and 6.1. Following inoculation of spores of *C. botulinum* type A into cheese spread with an estimated a_w of 0.970 and pH 5.7, growth and toxin formation occurred after incubation for 30–70 days at 30° C.[28] It was concluded that process cheese spreads could pose a safety hazard if subjected to temperature abuse (ie, at room temperature in tropical areas or during the summer), and that a method of reducing the hazard would be to lower the water activity of the product by the addition of components such as whey powder, NaCl, emulsifying salt (phosphate), and glycerol. The addition of potassium sorbate 0.3% w/w delayed toxin production by *C. botulinum* type A in a model system for cheese spread (apparently lacking in fat) and increased the margin of safety,[29] but in products with a high fat content this effect of sorbate would probably be reduced.

The inclusion of components such as vegetables and herbs in products of this type and any reduction of the concentration of NaCl and increase in the moisture content require an evaluation of the effects on safety with respect to *C. botulinum*.

41.9.10 Category 9. Perishable Products, *C. botulinum* Controlled by Mild Heat Treatment and Refrigeration; Ready Meals, "Sous-Vide" Foods

In northern European countries, there is a trend toward the increased production of precooked foods or meals for catering or for retail sale that are intended to have a shelf-life of up to 3 or 6 weeks at refrigeration temperature.[181,238] The safety of many of these foods with respect to *C. botulinum* depends mainly on a pasteurization heat treatment combined with refrigerated storage and a specified shelf-life and often they do not contain other preservative factors. The terms "Refrigerated, processed foods of extended durability (REPFEDs)" and "New generation refrigerated foods" have been applied to these products, but the former term, particularly, can apply to foods in categories 7 and 8 as well as to category 9 of Table 41–17. In the production of "sous vide" products, the food is packaged in heat-stable, air-impermeable bags under vacuum, the bags are sealed, and the product is cooked (pasteurized), cooled, and stored under refrigeration. In general REPFEDs are prepared by one of the following methods: (1) foods are packaged then cooked, or (2) foods are cooked then packaged, or (3) foods are cooked, packaged, then heated again.

These foods are given a much less severe heat treatment than are shelf-stable canned foods, with the aim of producing foods with a better flavor. The heat treatment, in the temperature range of 65° to 95° C, has little effect on spores of Group I, proteolytic strains of *C. botulinum* but damages or inactivates spores of Group II, nonproteolytic strains. It is essential, therefore, that throughout storage these foods should be maintained at a temperature below 10° C (ie, below a temperature that will allow growth of Group I strains).

In Europe, recommendations have been published that the heat treatments given to these foods or the combination of heat treatment with inhibitory factors should reduce the probability of survival and growth of nonproteolytic *C. botulinum* by a factor of 10^6 (ie, provide a protection factor [Pr] of 6). The UK Advisory Committee on the Microbiological Safety of Foods (ACMSF)[3,4] concluded that the safety of extended shelf-life chilled foods with respect to nonproteolytic *C. botulinum* could be ensured by one of the following:

- storage at <3.3° C
- storage at ≤5° C and a shelf-life of ≤10 d
- storage at 5 to 10° C and a shelf-life of ≤5 d
- a heat treatment at 90° C for 10 min or equivalent lethality followed by storage at <10° C
- a pH of <5.0 throughout the food combined with storage at <10° C
- an NaCl concentration of >3.5% throughout the food combined with storage at <10° C
- a water activity of <0.97 throughout the food combined with storage at <10° C
- other combinations of heat treatment and preservative factors together with storage at <10° C that can be shown consistently to give a protection factor of 6.

Similar guidelines have been issued by the European Chilled Food Federation (ECFF) and other organizations.[85,107,194] A draft code relating to these foods has been produced by the Codex Alimentarius.[53] It should be noted that growth and toxin production have now been reported at 3.0° C.[112]

There are very few circumstances under which it is possible to rely on maintenance of chilled foods at a temperature below 3.0° C for long periods of time (eg, > 5 days).[181] It is clear from surveys that while many firms are able to maintain these low temperatures in the distribution chain, during storage in retail conditions and by consumers such low temperatures cannot be maintained reliably.[258] The current regulations in Europe require chilled foods in general to be maintained at 0 to 3° C in Spain, 0 to 4° C in France, ≤7° C in Belgium, and ≤8° C in the UK.[238]

Many extended shelf-life chilled foods have a pH higher than 5.0, an NaCl content lower than 3.5%, and a water activity higher than 0.97. Thus the major factors that can be used to ensure the safety of these foods are the combination of heat treatment and maintenance of a storage temperature below 10° C, together with a specified shelf life. In foods that are packaged after the heat treatment, strict control of hygiene is necessary to prevent recontamination with pathogenic or spoilage bacteria.

Pasteurized, canned meat from the blue crab (*Callinectes sapidus*) has been produced in the US for many years. The standard practice, published in 1970, was reported in 1992 to involve heating cans of crab meat in a water bath at 88° C until cold-point temperatures reached 85° C for 1 minute. This process has been calculated to give an average heat treatment equivalent to 31 minutes at 85° C, assuming a z value of 8.9° C.[260] In conjunction with good manufacturing practices, experience in the industry indicated that this heat treatment gave a shelf-life for the product of 6 to 18 months, a storage temperature of 2.2° C or below being stipulated in the standards endorsed by the national Blue Crab Industry in the US.[260] The results described in Section 41.7.3 indicate that, unless blue crab meat causes an increase in the heat-sensitivity of spores of nonproteolytic *C. botulinum*, the heat treatment usually given to canned blue crab meat may give only a low level of inactivation of spores of nonproteolytic *C. botulinum*, many spores being sublethally damaged. This would mean that storage at a temperature ≤2.2° C is an integral factor required to ensure safety with respect to *C. botulinum*. Failure to maintain this low storage temperature could entail a risk that the sublethally damaged spores would germinate and result in growth and formation of toxin.

Tests to determine whether the pasteurization process for the blue crab could be applied to meat of the Dungeness crab (*Cancer magister*) showed that the heat process was inadequate to destroy 10^4 spores of *C. botulinum* type E and that toxin could be formed before the product showed signs of spoilage; thus the product would not be safe under conditions of temperature mishandling.[175] On the basis of end-point experiments, a process time of 50 to 56 minutes at 90.6° C or 7 to 17 minutes at 94.4° C was necessary to inactivate 10^6 spores of a mixture of three strains of nonproteolytic *C. botulinum* type B.[247] These processes are more rigorous heat treatments than the 90° C for 10 minutes proposed by the ACMSF and the ECFF for REPFEDs. As an extra precaution, the authors recommended storage of pasteurized Dungeness crab meat below 38° F (3.3° C).[247]

A series of publications has demonstrated combinations of heat treatment, pH, NaCl, storage temperature, and time that prevent growth and toxin formation from 10^6 spores of *C. botulinum* in a model food (meat slurry) (ie, that provide a protection factor of 6) and predictive models have been developed.[91,92,110,239,243] This work has shown, for example, that in a model food with added lysozyme, after heat treatment equivalent to 90° C for 34 minutes or 95° C for 15 minutes followed by storage at 25° C, growth occurred after 14 days and 32 days respectively; this indicates a heat resistance of a similar order to that reported in the Dungeness crab meat.[247] If, however, storage was at 12° C or lower, growth and toxin were not detected after 55 days.[92,243] Thus, although these heat treatments would not inactivate 10^6 spores in the presence of lysozyme, they might be used in combination with refrigerated storage and a limited shelf-life (eg, 42 days) to ensure the safety of food with respect to *C. botulinum* even if the food contained lysozyme.

Before commercial application, these results should be confirmed in specific foods, because foods may differ in their protective effects on spores during heating and may contain a higher concentration of enzymes with lysozyme activity than in these experiments. In some foods, the inclusion of a further controlling factor such as lower pH may be needed to obtain the required protection factor or to reduce the heat treatment or extend the shelf-life; the published information on the effect of combinations of factors[91,92,110,239,243] provides the basis for selecting an appropriate combination.

41.10 CONCLUDING REMARKS

The incidence of *C. botulinum* in some types of environment, such as some enclosed waterways, can be relatively high. In many parts of the world there is little information about the incidence of the organism; even where information is available, the data are relatively sparse. The improvements in methods of detection of the organism and its toxin, and the increasing availability of methods that do not depend on the use of mice, should facilitate studies that would give further information on the probable incidence of the organism in raw foods. This could be important if changes in production and sources of raw foods are likely to result in higher levels of contamination with spores of *C. botulinum* or other bacteria able to produce botulinum neurotoxin. The introduction of fish farming is an example of a change in production of raw food that has, in some conditions, been associated with an increased incidence of *C. botulinum*, and good practices have been published that prevent this problem.

In the case of many processed foods that have a good record of safety, the basis for that safety with respect to *C. botulinum* has not been fully evaluated. Studies to determine the basis of this safety are needed to ensure the continued safety of these products, particularly when gradual changes and variations in composition and processing are introduced.

Changes in methods of food processing and preservation will require evaluation to ensure that the required safety factor with respect to *C. botulinum* is built into the resulting foods. For example, the use of new methods of "commercial sterilization" such as treatment with high pressure and ohmic heating for low-acid foods must be designed to give at least the equivalent of the protection factor at present used for heat treatment of low-acid, canned foods.

The current rapid increase, in parts of Europe and North America, in sales of meals that rely for their preservation primarily on a combination of minimum heat treatment, refrigeration, and limited shelf-life entails a risk of growth of nonproteolytic *C. botulinum* (and of growth of proteolytic *C. botulinum* if products are subjected to temperature abuse). A substantial amount of recent research has been directed toward ensuring the safety of these foods.

Many of the outbreaks of foodborne botulism have been caused by the failure to apply information and safeguards that were well known and well understood by food microbiologists. There is clearly a need to ensure that commercial food producers, including large and small companies, and people who preserve food on a domestic scale, understand and apply the controls necessary to prevent foodborne botulism.

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42

Clostridium perfringens

Ronald G. Labbé

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42.1. INTRODUCTION

During the last 100 years, *Clostridium perfringens* has been most closely associated with clinical cases involving gas gangrene. The organism was first associated with food poisoning about 50 years ago and became more prominent following a 1953 paper by Hobbs et al.[103] Until the 1970s the organism was referred to as *Clostridium welchii.*

Although the symptoms associated with *C. perfringens* foodborne illness are relatively mild in healthy adults, deaths have often occurred in the elderly, in particular, those who were institutionalized. A particularly severe form of human intestinal disease caused by type C strains and known as "pig-bel" is discussed further in Section 42.3.2. A number of other disease states are associated with *C. perfringens* including septicemia, uterine infections, anaerobic cellulitis, sudden infant death syndrome, and enterotoxemia in a variety of domestic animals. This review will focus on food poisoning due to *C. perfringens* enterotoxin (CPE) produced by type A strains.

42.2. TAXONOMY AND CHARACTERISTICS OF THE ORGANISM

42.2.1 General

C. perfringens is a Gram-positive, anaerobic, spore-forming, encapsulated, nonmotile, rod-shaped bacterium. Under rapid-growth conditions (glucose-rich media), cells are shorter than in a slow-growth environment (starch-based media). The G-C content of the DNA is from 24 to 27 moles %. Based on analyses of 16S rRNA nucleotide sequences, the closest relative of *C. perfringens* identified to date is *Clostridium pasteurianum*.[31] Although an anaerobe, the organism will tolerate some exposure to air, thus facilitating the laboratory manipulations involved in its enumeration from food samples.

Sporulation is an important property of *C. perfringens* because it is a factor in classification, spores can survive cooking procedures and resume vegetative cell growth if proper conditions are present, and high levels of enterotoxin formation are associated with sporulation.

42.2.2 Toxin Typing

C. perfringens can produce at least 11 separate toxins in addition to an enterotoxin (Table 42–1), although each isolate produces only a specific subset of these. Details of each toxin have been described.[160,245] Strains are divided into five types based on the production of four extracellular toxins: alpha, beta, epsilon, and iota. The majority of strains produce α toxin (lecithinase, phospholipase C). The traditional method of toxin typing of strains involves the neutralization of the toxins with specific antiserum and determination of neutralization by injecting the toxin-antiserum mixture into mice or into the skin of guinea pigs.[191,245,248] The published sequences of the major toxin genes led Daube et al[45] to develop a toxin typing procedure based on the *in vitro* amplification of specific sequences of the major toxin genes. After electrophoresis, the pattern of bands corresponds to the specific toxin type. The chromosomal organization of these toxigenic types is relatively constant, suggesting that phenotypic differences (eg, extracellular toxins) are due to minor changes at the chromosomal level or from the acquisition of extrachromosomal genes.[30]

42.2.3 Serological Typing

In the 1950s, serological typing of the bacteria was first used to investigate outbreaks due to *C. perfringens*. The first 13 serotypes were prepared in the Food Hygiene Laboratory (London) by Hobbs et al[103] and were known as the "Hobbs types," a term still used today, eg, Hobbs 12 (H12). Cherniak and Henderson[33] provided a detailed analysis of the immunological relationships among serotypes 1, 5, 9, and 10. The isolated capsular polysaccharides formed a single precipitation line with their homologous antiserum.

In England antiserum production was almost entirely against outbreak strains, whereas in the United States (US) and Japan *C. perfringens* from a variety of sources, eg, environmental or fecal from healthy individuals, was used. The combination of 75 English, 34 American, and 34 Japanese antisera was used to serotype isolates of *C. perfringens* associated with outbreaks of food poisoning that occurred in the United Kingdom (UK) between 1970 and 1980. A specific serotype was implicated in 64% (of 646) of isolates using the English antiserum alone. Only an additional 5% of strains were typeable using the American and Japanese antisera; thus, only strains from confirmed outbreaks should be used for antiserum production.[252] The procedure for serotyping has been described by Stringer et al[253] who also listed four reasons for serotyping *C. perfringens*:

- to determine whether strains isolated from fecal specimens obtained from different individuals belong to the same serotype
- to determine whether isolates from the incriminated food and fecal specimens belong to the same serotype
- to establish the food involved when several foods have been consumed
- to identify related outbreaks.

Nevertheless, the development of a definitive serological typing scheme has been hindered by the antigenic heterogeneity of strains of *C. perfringens* type A. This has hindered epidemiological investigations. Other criteria exist for confirming the suspected role of isolates of this organism in outbreaks.

42.3 CHARACTERISTICS OF THE DISEASE

42.3.1 Gastroenteritis Caused by Type A Strains

Virtually all outbreaks of foodborne illness due to *C. perfringens* are caused by type A strains. Occasionally a lecithinase-negative strain is isolated from such outbreaks,[25,241] making the isolate, in principle, not *C. perfringens* (Table 42–2).

Typically symptoms of the illness occur 8 to 24 hours after ingesting food containing large numbers of vegetative cells, which have grown in temperature-abused foods—usually meat and poultry items. Diarrhea and severe abdominal cramps are the principal symptoms. Symptoms usually subside without treatment within 24 hours, although residual cramping may persist for an additional 24 to 48 hours. Vomiting and fever are uncommon. Mortality is low

Table 42–1 Soluble Antigens in *C. perfringens* Culture Filtrates

		Occurrence in Filtrates of C. perfringens Types[a]				
Designation	*Activity*	*A*	*B*	*C*	*D*	*E*
α	Lethal, necrotizing, hemolytic, lecithinase C	+++	+	+	+	+
β	Lethal, necrotizing	–	+++	+++	–	–
γ	Lethal	–	+	+	–	–
δ	Hemolytic, lethal	–	+	++	–	–
ε	Lethal, necrotizing (activated by trypsin)	–	++	–	+++	–
θ	Hemolytic (oxygen-labile, lethal)	++–	+	+	+	+
ι	Necrotizing, lethal (activated by trypsin)	–	–	–	–	++
κ	Collagenase (lethal, necrotizing, gelatinase)	++	+	+	+–	+
λ	Proteinase (disintegrates Azocoll and hide powder but not native collagen; gelatinase)	–	+++	–	+–	+
μ	Hyaluronidase	+–	+	–	+–	–
υ	Deoxyribonuclease	+	+	+	+	+
	Enterotoxin[b]	+	+	+	+	+

[a] +, ++, +++, produced by all strains
++– or +– –, present in some strains
–, not present in any strains

[b] in sporulating-cell extracts

Source: Reprinted with permission from J. Rood and S. Cole, Molecular Genetics and Pathogenesis of *Clostridium Perfringens, Microbiology Reviews,* Vol. 55, pp. 621–628, © 1991, American Society for Microbiology.

and generally occurs in the elderly. Undoubtedly the fact that symptoms are relatively mild has hindered proper epidemiological investigations of sporadic outbreaks, with only large-scale outbreaks prompting thorough investigation. The relatively brief nature of gastroenteritis probably occurs because the toxin preferentially binds to intestinal cells, which are rapidly replaced by normal turnover, and because the diarrhea associated with the illness causes the removal of unbound enterotoxin as well as toxin-containing sporulating cells. The illness is self-limiting and antibiotic therapy is not recommended in most cases. Dehydration, especially in the elderly, is the most important concern.

The enterotoxin responsible for foodborne illness is produced during sporulation of the organism in the small intestine, thus requiring the survival of ingested cells during stomach passage. It is unknown whether an acid tolerance response[59] is induced during this process; however, acid enhancement of sporulation by *C. perfringens* has been demonstrated in the case of at least one strain.[289] In any event it is likely that some lethality results from exposure to stomach acidity; hence the need for large numbers of ingested cells.

Table 42–2 Distribution of the Major Lethal Toxins among Types of *C. perfringens*

Type	*Alpha*	*Beta*	*Epsilon*	*Iota*
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

42.3.2 Related Illnesses

A more severe type of gastroenteritis caused by *C. perfringens* type C occurs among natives of the New Guinea highlands. It is a necrotizing, hemorrhagic jejunitis, commonly called pig-bel and associated with pig feasting.[279] About 70% of the villagers are carriers of the type C strains.[132] Malnutrition is an apparent risk factor because it leads to levels of intestinal proteases that are too low to destroy the toxin. Foods containing protease inhibitors, such as trypsin inhibitors, may prevent breakdown of the toxin in the gut.[131] The illness is rare in Western countries.

More recently a nonfoodborne, gastrointestinal illness of humans associated with *C. perfringens* has been associated with antibiotic use.[19,20] Most patients had received antimicrobials within 4 weeks before symptoms appeared and had high fecal counts of ent⁺ strains of *C. perfringens* as well as enterotoxin in their stools. The antibiotics may have disrupted the normal flora of the gastrointestinal tract sufficiently to allow small numbers of the ent⁺ strains to grow and produce enterotoxin. The situation is strikingly similar to antibiotic-associated diarrhea caused by *Clostridium difficile.*

There have been sporadic reports of foodborne illness due to *C. perfringens* with relatively short incubation periods (< 8 hours).[209,216,231] This suggests a role of preformed toxin or ingestion of sporulating cells. Given sufficient time, the organism can form large numbers of spores and enterotoxin in meat and poultry.[42,47,167,175] The likely adverse sensory qualities of the food in such cases[209] probably contributes to the rarity of such events. Emerging epidemiological evidence incriminates CPE in other human and veterinary disease apart from foodborne illness (Table 42–3).

42.4 MECHANISMS OF PATHOGENICITY

42.4.1 Histopathological Effects

Early studies regarding gastroenteritis caused by *C. perfringens* used viable cultures administered to animals and humans resulting in overt diarrhea. The purification of the enterotoxin led to the demonstration that it is the factor primarily responsible for experimental diarrhea in humans and in a number of animal models. These include pigs,[37] lambs,[93,183] sheep,[183,184,189] calves,[185,186] chickens,[187,188] dogs,[14] rats,[159,162,165] rabbits,[50,92,163–165,183,234,253] guinea pigs,[183] mice,[173,290] monkeys,[50,94,272] and humans.[256] Eight to 10 mg of purified CPE were required to induce symptoms in humans,[240] provided the gastric juice was neutralized with sodium bicarbonate.

The enterotoxin accumulates within the sporulating cells. Lysis of the cells releases the toxin, which then binds to epithelial cells. The brush border (microvillous membrane) of villous tip epithelial cells is the primary site of action of the enterotoxin. Histological studies of enterotoxin-treated ileal loops of rabbits reveal desquamation of epithelial cells at villous tips.[165] Tissue damage is detectable within 15 minutes of exposure of rabbit ileal loops to CPE.[227] The normal folded configuration of the brush border is lost and large quantities of membrane and cytoplasm are lost to the lumen. Scanning electron micrographs reveal localized morphologic damage to villous tips and a covering of rounded blebs on the tips (Figure 42–1). The toxin also causes "bleb" formation in rabbit villus epithelial cells (Figure 42–2) and in cultured Vero cells.[161] At least in the case of rabbits, the colon is relatively insensitive to the enterotoxin even though it binds well to rabbit colonic cells.[161] The sensitivity of the rabbit's small intestine to the enterotoxin increases from the upper duodenum downward, with the terminal ileum being the most responsive.[164] Although mode of action studies of CPE-induced alterations of morphology have used Vero (African green monkey kidney) cells, similar changes occur in mammalian intestinal epithelial cells.[155]

Table 42–3 Human and Veterinary Diseases Associated with *C. perfringens* Enterotoxin

Illness	*Reference*
Human food poisoning	123, 157
Antibiotic-associated diarrhea of humans	19, 86
Chronic nonfoodborne diarrhea	157
Acute sporadic (nonfoodborne) human diarrhea	25, 130, 174
Sudden infant death syndrome	136–138, 143
Animal diarrhea	37

Source: Adapted with permission from B. McClane, *Clostridium Perfringens* Enterotoxin Acts by Producing Small Molecule Permeability Alterations in Plasma Membranes, *Toxicology*, Vol. 87, pp. 43–67, © 1994, Elsevier Applied Science.

42.4.2 Molecular and Cellular Effects

The development of an *in vitro* model system for studying the enterotoxin has led to an understanding of its mode of action at the cellular level. Vero cells have been used widely for these studies, although other cell lines also have been used as models.[107,110,161,242] By contrast, Chinese hamster ovary cells are insensitive to the enterotoxin.[283] Structure and function mapping studies of CPE have identified the N-terminal region as involved with insertion and cytotoxicity and the C-terminal end with binding (Figure 42–3).[152] Following detailed studies of the effect of the enterotoxin, McClane and coworkers established the sequence of events in the action of the toxin (Figure 42–4). The initial calcium-independent event involves binding of the toxin to its receptor, a 50 kDa membrane protein.[286] In contrast to the situation with *Escherichia coli* labile enterotoxin and *Vibrio cholera* enterotoxin, no role has been identified for ganglioside GM_1 from rabbit intestinal brush border membranes in the binding of *C. perfringens* enterotoxin.[286] The CPE forms a large complex with membrane proteins and remains bound to the membrane without entering the cell.

CPE initially causes cells to become permeable to the influx and efflux of a variety of molecules of less

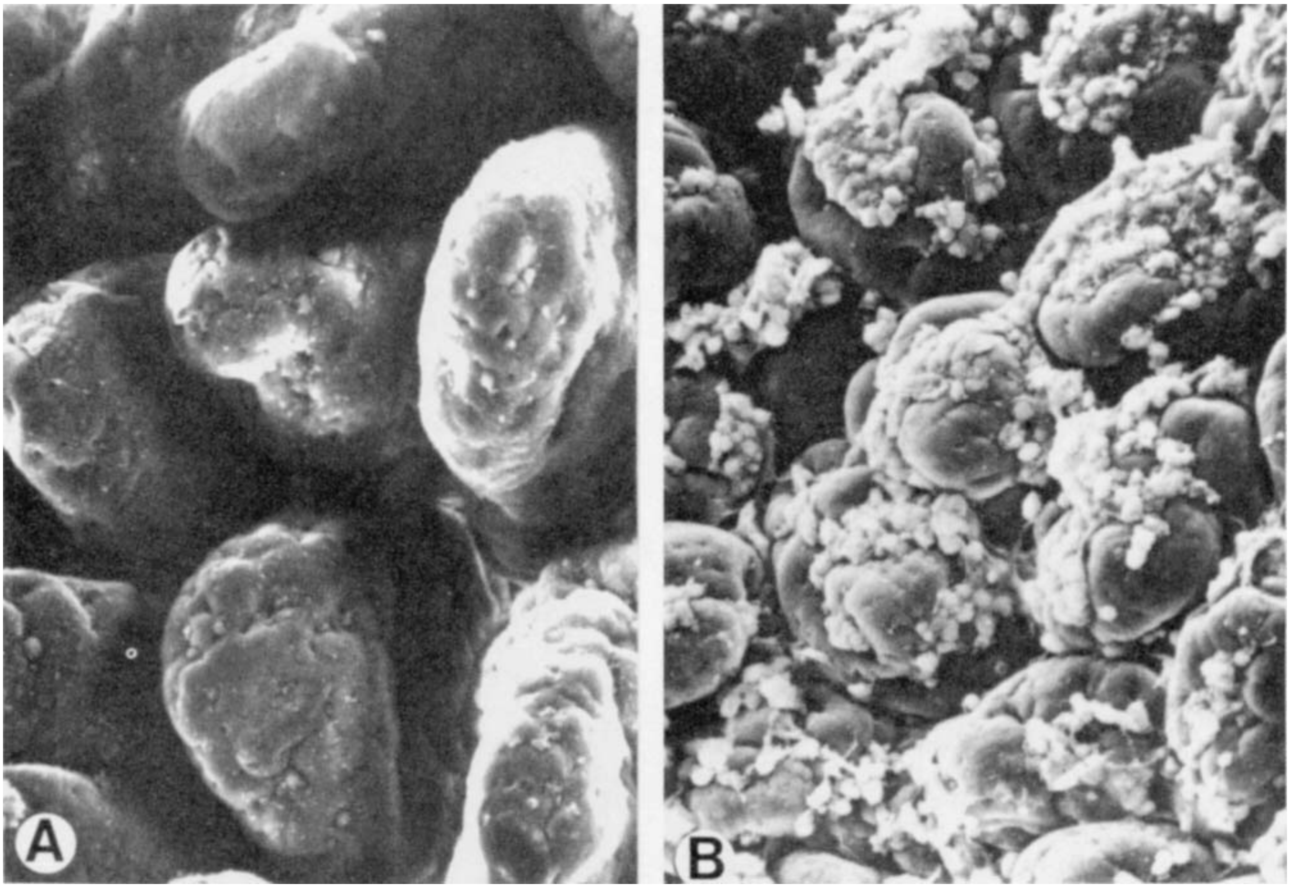

Figure 42–1 Effect of *C. perfringens* Enterotoxin on Rabbit Ileum. **A.** Scanning electron micrograph of control showing typical villus morphology, x 370. **B.** Treated with CPE for 90 minutes showing collapsed villus tips and protruding structures at the surface of epithelial cells, x 250. Samples were fixed in glutaraldehyde and osmium textroxide and coated with 20 to 30 nm of gold palladium. *Source:* Reprinted with permission from J. McDonel et al., The Effects of *Clostridium Perfringens* Enterotoxin on Rat and Rabbit Ileum, *Laboratory Investigations*, Vol. 39, pp. 210–218, © 1978, Lippincott Williams & Wilkins.

than 200 daltons, such as ions and amino acids,[149,151,153] perhaps by the formation of pore structures. Osmotic stabilizers protect against such CPE-induced permeability changes.[151,156] Events after small molecule permeability alterations appear to be secondary consequences and include inhibition of DNA, RNA, and protein synthesis, and bleb formation.[155] Water follows the ion influx into the cytoplasm causing the plasma membrane of treated cells to stretch, forming blebs. The change in permeability to small molecules leads to the loss of macromolecular precursors and loss of viability. In cases of foodborne illness, this presumably leads to intestinal cell lysis and desquamation of intestinal cells from villous tips. Loss of fluids and electrolytes corresponds to the diarrheal symptoms associated with the illness. The molecular events associated with CPE action on mammalian cells have been recently described in detail.[153,154]

42.4.3 Characteristics of the Enterotoxin

The enterotoxin was purified in the early 1970s and its amino acid sequence subsequently determined.[44,154,275] In the case of strain NCTC 8798, the toxin is composed of a single polypeptide with a unique sequence of 319 amino acids with a molecular weight of 35,317 and a pI of 4.3.[44,152] The toxin aggregates from solution at concentrations above 0.5 mg ml^{-1} at 4° C, perhaps due to its high hydrophobic amino acid content (43%).[73]

The enterotoxin loses its activity after treatment with pronase and subtilisin.[160] Limited exposure to trypsin and chymotrypsin actually increased its biological activity,[70,75] suggesting its possible activation in the small intestine during foodborne illness. A loss of 36 and 25 amino acids from the N-terminus occurs in the case of chymotrypsin and trypsin treatment, respectively. The toxin is also easily inacti-

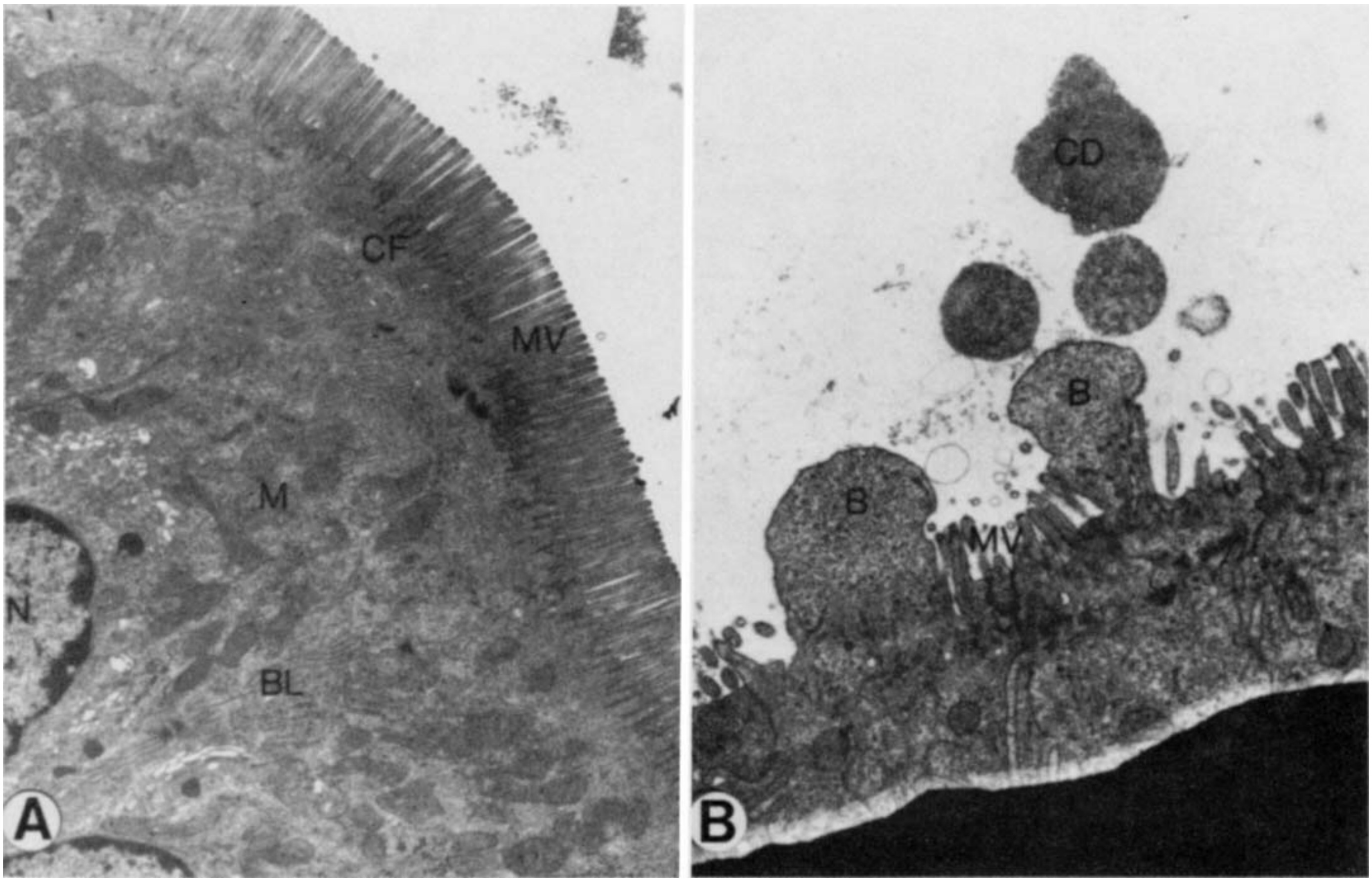

Figure 42–2 Effect of *C. perfringens* Enterotoxin on Rat Ilium. **A.** Transmission electron micrograph of control showing normal villus epithelial cell morphology; MV, microvilli. **B.** Treated with CPE for 90 minutes showing bleb formation × 11,900. Samples were fixed in glutaraldehyde and osmium tetroxide, and stained with uranyl acetate and lead citrate. *Source:* Reprinted with permission from J. McDonel, The Molecular Mode of Action of *Clostridium Perfringens* Enterotoxin, *American Journal of Clinical Nutrition*, Vol. 32, pp. 215–216, © 1979, American Society for Clinical Nutrition.

vated by heat. In saline, exposure to 60° C for 5 minutes destroys biological activity.[177] Others have reported a 90% loss in biological activity within 1 minute at 60° C in buffer, pH 6.8.[71] Serological activity is affected more slowly, with 20% remaining after heating at 60° C for 20 minutes. Serological activity is lost more rapidly at pH <6.0. In food samples, about 12% of serological activity destroyed by heating at 60° C can be restored by treatment with urea at room temperature for 1 hour.[177] Other workers reported that CPE, heated at 55° C for 1 minute (70% loss of activity) regained 50% of original biological activity after 4 days.[71] CPE stored at 37° C for 7 days or at –20° C loses biological but not serological activity;[167] however, neither biological nor serological activity is affected by storage at 4° C for several weeks in 0.02 M phosphate buffer, pH 6.8.[75] About 5% to 10% of the toxin's original activity is lost each time it is thawed; therefore, lyophilization is the preferred method of storing CPE.

Maximum ultraviolet absorption occurs at 276 nm. The extinction coefficient at pH 6.8, $E_{1\mathrm{cm}}^{0.1\%}$ at 276 nm, of CPE, is 1.33.[74] In 0.02 M phosphate buffer, pH 6.8, its maximum solubility at 25° C is 3.94 ± 0.22 mg ml^{-1}.[74] Enterotoxin has also been detected in *C. perfringens* types B, C, and D.[147,239,273] Its properties are similar to those of enterotoxin of type A strains.

There have been few studies on the immune response to CPE. Like staphylococcal enterotoxin A, CPE is a superantigen that stimulates large quantities of T-cells,[21] although it is not known whether this is linked to enterotoxicity or cytotoxicity. Analysis of deletion mutants indicates that the minimum size CPE fragment that retains cytotoxicity appears to be CPE amino acids 45 to 319.[118]

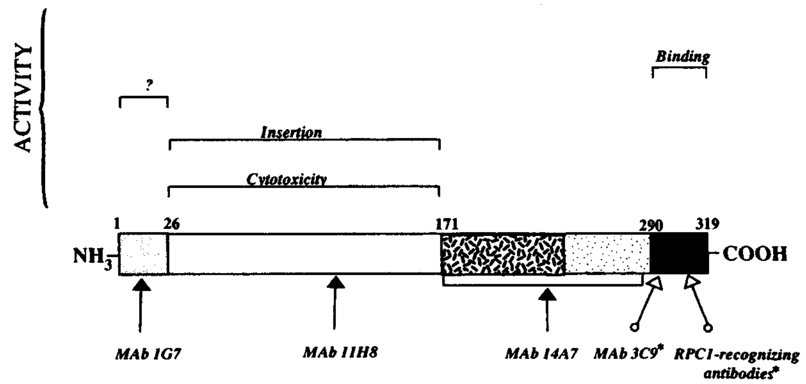

Figure 42–3 Map of *C. perfringens* Enterotoxin Functional Regions, Showing Regions Involved in Insertion, Cytoxicity, and Binding. Also shown (arrows) are sequences required for presentation of epitopes recognized by various monoclonal antibodies (Mab). Asterisks designate antibodies that neutralize biological activity. RPC1, rabbit polyclonal anti-CPE serum. *Source:* Reprinted with permission from B. McClane, *Clostridium Perfringens* Enterotoxin Acts by Producing Small Molecule Permeability Alterations in Plasma Membranes, *Toxicology*, Vol. 87, p. 43–67, © 1994, Elsevier Applied Science.

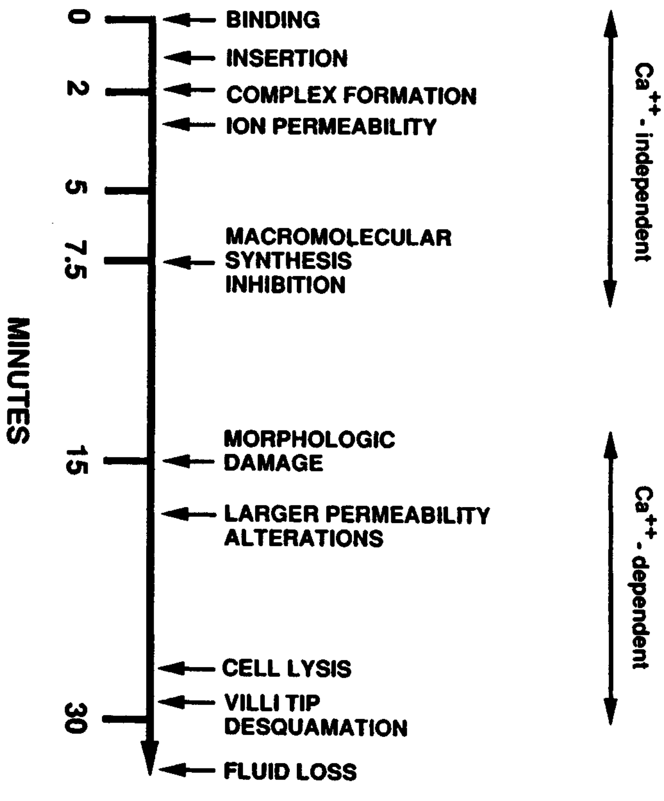

Figure 42–4 Time Line for Events in Action of *C. perfringens* Enterotoxin. It is now known that the calcium-dependent events start after approximately 8 minutes. (McClane, pers. commun.) *Source:* Reprinted with permission from B. McClane, *Clostridium Perfringens* Enterotoxin: Structure, Action, and Detection, *Journal of Food Safety*, Vol. 12, p. 244, © 1992, Food & Nutrition Press, Inc.

42.4.4 Production and Isolation of *C. perfringens* Enterotoxin

In vitro Synthesis

In the laboratory, crude enterotoxin is obtained from extracts of late-stage sporulating cells. Many strains notoriously are reluctant to sporulate and sporulation is distinctly strain- and medium-dependent. Cooked meat medium (CMM) is commonly used for growth of the organism, and stock cultures can be maintained frozen in this medium for many years. The level of sporulation in CMM is insufficient for high CPE yields and the medium of Duncan and Strong,[49] or minor modifications of it,[40,84,112,270] have been the most widely used. The medium is composed of yeast extract, peptone, starch, sodium thioglycollate, and sodium phosphate. Replacing the starch with raffinose increases the sporulation and CPE levels of some strains.[128,270] In the presence of raffinose, the incubation temperature can be increased from the standard 37° C to 43° C. This shortens (by about 2 hours) the time required for (but not the extent of) maximum levels of sporulation and CPE formation.[61] Certain methylxanthines (caffeine, theobromine, and theophylline)[112,127,225] and bile salts[98] also can enhance the level of sporulation. Other factors that may affect the level of sporulation include the use of overnight vs log-phase cultures and the amount of inoculum. These must be determined empirically; a single set of conditions may not promote sporulation by all strains.

Several solid sporulation media also have been described.[247,269] These could be useful for the direct detection of sporulating, enterotoxin-producing colonies, eg, by enzyme-linked immunosorbent assay

(ELISA); however, the availability of colony hybridization techniques using DNA probes for the *in situ* detection and enumeration of vegetative cells carrying the *cpe* gene[9] has diminished the need for solid sporulation media.

Certain situations require a defined sporulation medium; for example, metabolic studies of the sporulation process,[48] purification of extracellular products produced during sporulation,[192,193,233] or radioactive labeling of CPE.[72,141] In addition, the nutritional requirements for sporulation and enhancers of sporulation, such as methylxanthines, can be more accurately assessed using a defined medium.[121] For these purposes, the defined sporulation medium of Sacks and Thompson[224,226] is quite suitable, although as with the complex media, not all strains respond with a high percentage of sporulating cells. This medium and other defined media[66,207,233,264] are based on earlier formulation by Boyd et al[22] for vegetative cell growth.

The pattern of CPE formation during growth in a complex medium is now well established.[120,251] In defined media, the kinetics are similar but the lag phase is longer [Labbé, unpublished]. Briefly, at 37° C, heat-resistant spores appear after between 3 and 4 hours and increase rapidly, reaching maximum numbers after 7 hours, typically between 2×10^7 ml^{-1} and 5×10^7 ml^{-1}, remaining at that level for at least 48 hours. Intracellular CPE formation (measured as a proportion of cell extract protein) parallels spore formation decreasing after about 10 hours as sporangial autolysis occurs. It is at this point that CPE is detectable in the culture fluid. In cases of foodborne illness, this would correspond to its release in the lumen of the small intestine and subsequent action on microvilli.

The pattern of *cpe* gene transcription and translation during sporulation is shown in Figure 42–5. During exponential growth no CPE protein can be detected and only trace amounts of *cpe* mRNA are seen. The induction of *cpe* mRNA and CPE protein occurs simultaneously about 1 hour after the end of exponential growth (T_0) and rises sharply throughout stationary phase. This indicates that the induction of *cpe* gene expression is an early stationary-phase event. This is in line with the earlier conclusions of Duncan et al[52] who isolated Spo$^-$ mutants and showed that mutants blocked at stage 0 fail to make CPE, while those blocked at later stages, III to V, were able to do so. Thus it is likely that *cpe* gene expression is induced after stage 0 and before stage III (no stage II mutants were isolated).

Some strains of *C. perfringens* that form high concentrations of enterotoxin form an inclusion body in the sporangium (Figure 42–6);[53,262] this is composed of CPE and has biological activity.[139,140] This aggregated CPE is in addition to soluble toxin present in cell extracts. Its role in human illness is unclear.

Purification of Enterotoxin

Of the methods proposed for the purification of CPE, that using ammonium sulfate precipitation of sporulating cell extracts followed by gel filtration[74] has been most widely used. The soluble, intracellular protein concentration of several sporulating strains can consist of up to 30% to 40% CPE,[128] although for most outbreak strains the level is about half this amount. The use of such strains together with growth at 43° C has yielded a protocol for CPE purification within 48 hours.[97]

Occasionally some toxin preparations contain a minor band after purification[12,55,229] as visualized by polyacrylamide gel electrophoresis (PAGE). This minor band possesses weak biological and serological activity. The molecular weight, but not the charge, is the same as that of the major band, perhaps due to endogenous protease activity.[139,195] The use of the protease inhibitors EDTA and phenylmethylsulfonylfluoride and low temperatures prevents such artifacts.[97,195]

The efficacy of protein purification protocols is confirmed usually by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE). It has been shown repeatedly that this procedure results in the aggregation of CPE with the appearance of several higher molecular weight bands.[43,56,69,106,158,205,223,230] However, no aggregation of the CPE is seen below 15 µg ml^{-1}.[222] PAGE, under nondenaturing conditions, does not result in such anomalous aggregation.

Relationship of Enterotoxin Formation to Sporulation

The pioneering work of Duncan et al. in *C. perfringens* research included the isolation of mutants of an enterotoxin-positive strain blocked developmentally at various stages of sporulation.[52] Sporulation stages are numbered from 0 (vegetative cell) to VII (sporangial autolysis). The ability or inability of these mutants to produce CPE originally led to the conclusions that CPE appears between stages 0 and III and it is a sporulation-specific gene product. A close correlation has long been known between the intracellular accumulation of CPE and sporulation; nevertheless, subsequent studies using serologic techniques demonstrated low levels of CPE in vegetative cells of *C. perfringens*.[65,76] This could have resulted from reactivity to a 48-kDa,

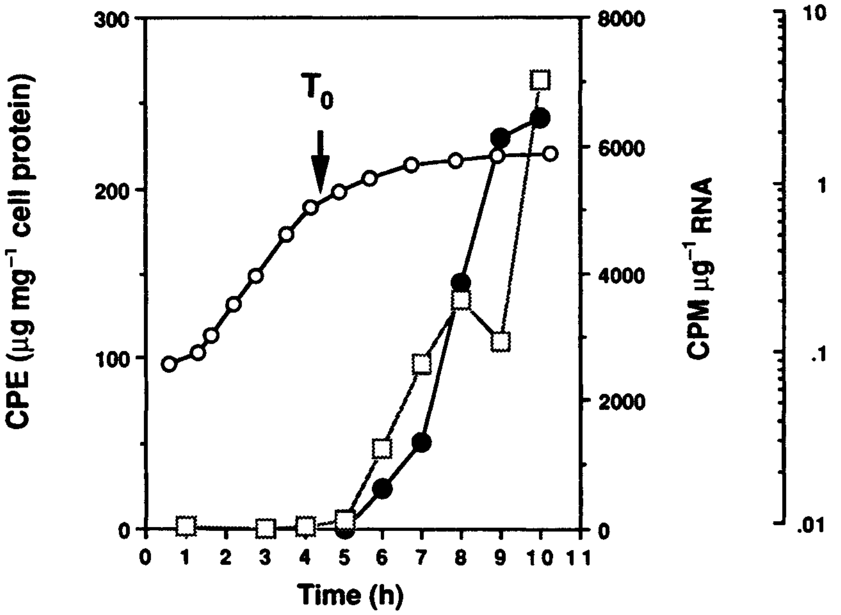

Figure 42–5 Synthesis of *cpe* mRNA (Squares) and *C. perfringens* Enterotoxin Protein (Solid Circles) during Growth (OD_{600} [Open Circles]) of *C. perfringens* NCTC 10240 at 37° C in Modified Duncan-Strong Sporulation Medium. *Source:* Reprinted with permission from S. Melville, R. Labbé, and A. Sonenshein, Expression from the *Clostridium Perfringens* CPE Promoter in *C. Perfringens* and *Bacillus subtilis*, *Infection and Immunity*, Vol. 62, p. 5554, © 1994, American Society for Microbiology.

CPE-related protein that has been shown to exist in vegetative cells;[223] however, using a more sensitive Western blot assay, Czeczulin et al[44] did detect exceedingly small amounts of CPE in cell extracts of ent+ vegetative cells. No biological activity was detected. Further, cell lysates from recombinant *cpe*-positive *E. coli* were shown by quantitative Western blot analysis to contain moderate amounts of CPE.[44]

The CPE content in sporulating cells can amount to a significant portion of the soluble cell extract protein in certain strains,[61,121,128] but knowledge of the function of CPE remains elusive. Earlier proposals that CPE may serve as a structural spore coat protein appear unlikely because relatively small amounts of CPE are associated with the spore, perhaps as a result of having been "trapped" during spore development.[222] Furthermore, virtually all enterotoxin-negative strains are capable of sporulation. It seems more likely that the *cpe* gene plays no role in sporulation but is activated by transcriptional factors that also control sporulation genes. Studies of *cpe* regulation at the transcriptional level may provide an explanation for the high levels of CPE produced during sporulation. In summary, very low levels of enterotoxin may be formed by vegetative cells, but high concentrations are produced only in association with sporulation, and this is unquestionably involved in causing foodborne illness.

Genetics of Enterotoxin Formation

Recent years have seen the cloning, sequencing, and other genetic studies on *C. perfringens* toxins, including α toxin, θ toxin, ε toxin, ι toxin, κ toxin, β toxin, sialidase, and hyaluronidase.[220] These have been used in vaccine and virulence studies. The physical map of one *C. perfringens* strain (CPN50) now comprises almost 100 markers.[34,36,117] The complete *cpe* gene from two CPE-positive strains has been cloned.[38,44] The availability of *E. coli-C. perfringens* shuttle plasmids,[10,214,243] reporter systems,[28,150,200] methods for electroporation-induced transformation,[6,232] and other genetic methods have shown that CPE synthesis is regulated at the transcriptional level. The *cpe* gene was found at the same chromosomal locus in three strains associated with food poisoning;[38] whereas, in 20 strains originating from domestic livestock the *cpe* gene was located on a large episome. This suggests the possibility of intergeneric transfer and, in fact, conjugation and mobilization of large plasmids in *C. perfringens* is well documented.[24,219] Similar to the variation in location of the *cpe* gene, two types of promoter regions have been reported to exist.[171] The cloning and sequencing of the entire *cpe* structural gene and its location on the restriction map of the *C. perfringens* chromosome[30] should result in the rapid elucidation

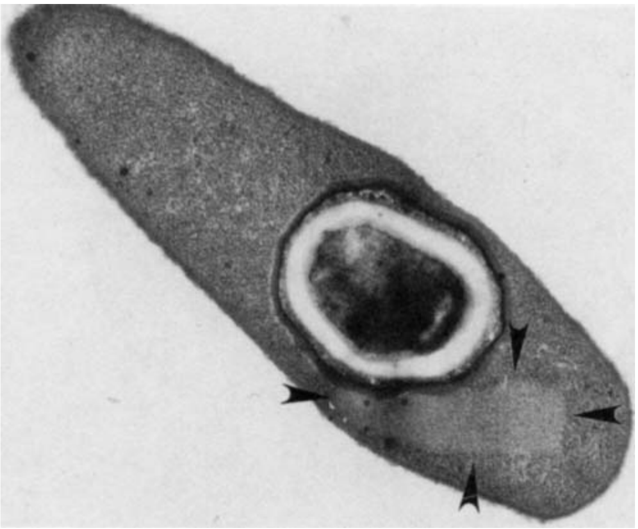

Figure 42–6 Transmission Electron Micrograph of *C. perfringens* Showing Inclusion Body during Sporulation of Strain NCTC 8679, × 47,000. Cells were grown at 37° C in a defined sporulation medium and fixed and stained as in Figure 42–2. *Source:* Reprinted with permission from A. Loffler and R. Labbé, Isolation of an Inclusion Body from Sporulating, Enterotoxin-Positive *Clostridium Perfringens type A, FEMS Microbiology Letters,* Vol. 27, p. 145, © 1985, Elsevier Applied Science.

of factors controlling the expression of the *cpe* gene. The reader is referred to recent articles detailing the molecular genetics of *C. perfringens* in general[34,35,142,218,219] and CPE in particular.[73,172]

42.5 INCIDENCE OF THE ORGANISM

42.5.1 In the Environment

Types B, C, D, and E can be considered parasites of domestic animals and do not survive in soils, dying out within a few months if added to soil samples, presumably because they are unable to compete with type A strains and other microflora. By contrast, type A strains are part of the microflora of both soil and intestinal tracts. Their numbers in soil are estimated to be several thousand per gram. The organism can be found also in low numbers in marine sediments, those present probably representing recent pollution.[148,227]

42.5.2 In Foods

Numerous surveys have been conducted on the incidence of *C. perfringens* in raw and processed foods; the results of a number of these are shown in Table 42–4. Raw protein foods of animal origin are far more likely to contain vegetative cells or spores of the organism than are processed foods. These reports indicate widespread occurrence of the organism in meat and poultry. About 50% of raw ground beef contains the organism.[60,129] *C. perfringens* was isolated from 29%, 66%, and 35% of beef, pork, and lamb carcasses, respectively,[244] although the numbers, obtained adjacent to the anus, were surprisingly low (< 20 per 100 cm^2). Intestinal tissues prevent the release of bacteria into the body cavity for several hours after death,[64] indicating that blood and lymph vessels do not normally offer a pathway for microbial invasion from the intestine. In addition, adequate rest and starvation before slaughter can dramatically reduce internal contamination of carcasses.[181]

Although not commonly involved in foodborne illness, fish (body surfaces and alimentary canal) were found positive for *C. perfringens.*[264] In a survey of vacuum-packed fish products, 67% were positive for clostridia, predominately *C. perfringens.*[102]

C. perfringens was isolated from 80% of 54 different spices and herbs.[46] The presence of this organism in herbs and spices, especially in the spore state, creates a hazard because such products are added often to large amounts of meat and poultry. Spores can survive cooking, and slow cooling could result in the growth of large numbers of vegetative cells.

Most surveys of the incidence of *C. perfringens* have not identified the enterotoxin-producing potential of the isolates. This is increasingly important because, in the case of nonsymptomatic animals, only 6% of strains possessed the *cpe* gene.[276] Only about 60% of food and fecal isolates (from 186 outbreaks) hybridized with synthetic DNA probes for the *cpe* gene,[277] suggesting that strains that could not produce CPE had been isolated originally.

42.5.3 In Feces

Up to 39 different *Clostridium* species have been isolated from human feces with *C. perfringens*, typically enterotoxin negative, being the most common isolate.[63] Some early studies focused on the heat-resistant *C. perfringens* strains (those whose spores survive 100° C for 1 hour). Surveys of the *C. perfringens* levels, regardless of type of spore heat resistance, indicate that most adults harbor the organisms, with median counts typically in the range of 10^3 to 10^4 g^{-1} feces;[91,251,260] however, spore concentrations of up to 10^9 g^{-1} (median: 10^5 g^{-1}) have been reported in healthy adults from Japan and from the elderly in Japan and the UK.[5,178,251] Such high counts

Table 42–4 Incidence of *C. perfringens* in Retail Foods

Product	*Number Samples Examined*	*% Positive*	*Levels*	*Reference*
Veal	17	82	most between 1 to 100 g^{-1}	78
Beef	50	70		78
Chicken	26	58		78
Pork	27	52		78
Processed meats	101	20		78
Frozen foods	111	3	10 to 20 g^{-1}	255
Raw fruits and vegetables	52	4	10 to 140 g^{-1}	255
Spices	60	5	10 to 30 g^{-1}	255
Meat, poultry, fish	122	16	10 to 1180 g^{-1}	255
Spaghetti sauce/mixes	13	54		179
Sauce and gravy mixes	8	12		179
Soup mixes	28	4		179
Cheese and cheese sauces	6	17		179
Bovine livers	100	26		29
Poultry livers	11	36		134
Poultry gizzards	11	46		135
Spices/herbs	147	59	10^2 to 10^4 g^{-1}	46
Spices	2	100	2.0×10^3 g^{-1}	182
Pork sausage	18	39	5 to 95 g^{-1}	15
Frozen, precooked meat/poultry	32	50		267
Raw ground beef	150	56	mean:55 g^{-1}	60
Raw ground beef	95	47	mean:5 to 100 g^{-1}	129
Vacuum-packed fish	140	23		102
Frozen chickens	118	63		210
Beef carcass	100	29		244
Pork carcass	100	66	20 100 cm^{-2}	244
Lamb carcass	100	85		244

have been found also in patients with infectious diarrhea not associated with food poisoning.[130] A detailed review of the clostridial flora in humans was presented by George and Finegold.[63]

In Europe the presence of *C. perfringens* has been used to supplement water quality information,[192] and the World Health Organization (WHO) has recommended it as a useful indicator of fecal pollution of water.[108] Fecal coliforms are poor indicators of the presence of human bacterial viruses in drinking water. *C. perfringens* is a suitable indicator for the inactivation and removal of viruses in drinking water[196] in particular and for evaluating the effectiveness of wastewater and sludge treatment systems in general.[101] Finally *C. perfringens* (presumably in the spore state) was shown to be a useful indicator of distribution of sewage sludge on the ocean floor[99] where conditions (2° C, 250 atmosphere [25.3 MPa]) are presumably unsuitable for coliforms and fecal streptococci. These applications have been facilitated by the development of rapid membrane filtration techniques for detection and isolation, combining high incubation temperatures, biochemical characterization, and antibiotics.[8,18]

42.6 EPIDEMIOLOGY

C. perfringens food poisoning is a worldwide problem, present wherever meat and poultry, in particular, are consumed regularly. The incidence is widely under-reported due to the perceived mild nature of the illness and differences between countries in their surveillance arrangements (Table 42–5). The number of outbreaks reported in England and Wales, where surveillance of this disease generally is more closely integrated, albeit informally, far exceeds that in the US, where reporting of this disease is largely uncoordinated nationally. The small number of confirmed outbreaks from the US in 1987 highlights the lack of vigilance and low priority associated with this illness by public health authorities of one country alone. WHO, as part of its Surveillance Program for the Control of Foodborne Infections and Intoxications in Europe, collects data from outbreaks in many countries; however, for most countries *C. perfringens* is not specifically reported.

As mentioned above, meat and poultry are the most common vehicles; given the organism's fastidious growth requirements and its presence in these

Table 42–5 Reported Incidence of Confirmed *C. perfringens* Foodborne Illness in Selected Countries

	Canada[a]		*United States*[271]		*Japan*[b]		*England and Wales*[c]		*Croatia*[206]	
Year	*Outbreaks*	*Cases*	*Outbreaks*	*Cases*	*Outbreaks*	*Cases*	*Outbreaks*	*Cases*	*Outbreaks*	*Cases*
1983	–	–	5	353	16	4,571	68	1,624		
1984	20	888	8	882	12	1,733	68	1,716		
1985	13	390	6	1,016	9	971	64	1,466		
1986	21	354	3	202	22	3,258	51	896		207
1987	18	369	2	290	9	288	51	1,266		359
1988	42	456	—	—	19	2,671	57	1,312		300
1989	13	381	7	436	24	3,316	55	901		281
1990	4	84	11	1,240	24	2,503	53	1,442		62
1991			10	1,213	21	3,691	44	733		328
1992			12	912	17	1,086	36	805		171
1993					9	1,077	38	562		
1994					16	1,821				

[a] E. Todd (personal communication).

[b] T. Uemura, (personal communication).

[c] R. Gilbert (personal communication).

products, this is not surprising. Food service establishments such as restaurants, hospitals, factories, prisons, schools, and caterers are the most likely sources of acquiring the illness. This results from needing to cook large amounts of food well in advance of serving.

42.7 FACTORS AFFECTING SURVIVAL IN FOOD

The two outstanding characteristics of *C. perfringens* that invariably contribute to its role in foodborne disease outbreaks are its rapid growth at temperatures of 43° to 46° C and its spore-forming ability. Factors affecting the growth and survival of the organism are dependent on whether the vegetative cell or spore is being considered; thus, each cell form will be considered separately.

42.7.1 Vegetative Cells

Temperatures for Growth

C. perfringens is able to grow in the range of 15° to 50° C. Growth at 15° C in aerobic or vacuum-packed cooked beef occurred only after several days,[39,114] but other workers have shown that *C. perfringens* (ent$^+$ strains) can grow in cooked roast beef at 12.8° C (55° F) when coinoculated with *Pseudomonas fragi*, presumably due to oxygen consumption by *P. fragi*.[100] A modified atmosphere (75% CO_2, >2% O_2, balance N_2) prevented growth of *C. perfringens* at 12.8° C (but not at 26.7° C) when coinoculated with *P. fragi*. Modified atmosphere packaging cannot be used effectively at room temperature to inhibit *C. perfringens* and cannot be used as a substitute for refrigeration.

Unique among foodborne pathogens, *C. perfringens* has a relatively high optimum growth temperature, 43° to 46° C, depending on the strain, although Willardsen et al[285] described one ent$^+$ strain as having a slightly shorter generation time (G_t) at 41° C than at 45° C. No lag was reported at 46° C for cells grown in meat although at 35° C there was a 2 to 4 hour lag period.[79]

G_t of approximately 9 minutes have been reported for ent$^+$ strains or for a composite of ent$^+$ and ent$^-$ strains growing in raw or cooked ground beef.[126,284,285] These times are among the shortest for any bacterium, and the ability for such rapid division has obvious public health importance. Even at 37° C one ent$^+$ strain had a G_t of 10 to 12 minutes in cooked chicken or turkey, while the same composite of strains had an average G_t of 30.8 minutes in cooked ground beef at 37° C.[285]

Few strains of *C. perfringens* grow at 49° C[79] and no growth has ever been reported at 52° C or above. However, apparent thermal adaptation by this organism can occur because each of two ent$^+$ strains showed more than a two-fold increase in $D_{57° C}$ value when the growth temperature was increased from 37° C to 45° C.[221]

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The literature up to 1979 describing the effects of temperature on growth has been reviewed by Craven.[39] The important points that emerge are that at static temperatures between 33° C and 49° C, G_ts of < 20 minutes can be expected for *C. perfringens* growing in food and that vegetative cells are inactivated readily at 55° C and higher. It is unknown whether the generation times of ent$^+$ strains as a class differ from those of ent$^-$ strains. Labbé and Huang[126] were unable to establish such a difference, although only two strains from each group were used.

In comparison with growth at static temperatures within the optimum temperature range, the possibility of growth during cooling has received less attention. To minimize microbial hazards, government agencies specify cooling parameters for food. For example, the U.S. Food and Drug Administration recommends all food be cooled from 60° C to 5° C in 6 hours or less.[275] No growth of a three-strain composite of spores of *C. perfringens* occurred in pasteurized, cooked beef cooled from 54.4° C to 7.2° C in 15 hours or less,[115] although a several log increase did occur if the cooling period was extended to 18 hours.

Rapid growth of two ent$^+$ strains of *C. perfringens* occurred at 28° C in anaerobically packaged cooked, ground turkey, with the numbers increasing by more than 5 log cycles within 9 hours[113] or, in the case of ground beef, by almost 3 log cycles within 18 hours (no increase after 12 hours).[111] On the other hand, refrigerated (4° C), aerobically or anaerobically packaged cooked ground beef temperature-abused at 28° C for 6 hours did not support the growth of *C. perfringens.*[111] Similarly, no growth from spores occurred within 150 minutes in cooked, ground beef inoculated at 60° C and cooled to 15° C at a falling rate of 15° C per hour.[235]

Taken together, the results suggest that *C. perfringens* may grow to unsafe levels in foods, including *sous vide* products, if such foods are temperature-abused for relatively long periods, but short periods of temperature abuse of refrigerated meat do not necessarily result in hazardous cell levels.

Low-Temperature Storage

C. perfringens is notably sensitive to low-temperature storage.[255,266] Stationary-phase cells are more resistant to the effect of cold shock than log-phase cells, which were reduced by 80% to 90% within 60 minutes when held in 0.1% peptone at 4° C.[266] Similarly, more than 90% of cells were inactivated following frozen storage at -17.7° C for 30 days in a starch paste, and even more in buffer.[258] Other studies of *C. perfringens* in food products held at -20° C for 17 days or at -29° C for 28 days resulted in a survival rate of 0.1% and <1% respectively.[82,266]

Oxidation-Reduction Potential (E_h)

C. perfringens will not produce colonies on agar exposed to air. In liquid media, the E_h is maintained at suitable levels by removal of oxygen and the inclusion of reducing agents such as cysteine and sodium thioglycollate, and by the use of low concentrations of agar to minimize oxygen diffusion. Simply steaming liquid media for a few minutes is effective in removing oxygen. Multiplication of *C. perfringens* was inhibited in the presence of oxygen in both the negative and positive ranges of E_h;[278] however, in the absence of oxygen multiplication occurred at both negative or positive E_h. The E_h levels of laboratory media are typically from +200 mV to +250 mV.[11,169] The upper limit that inhibits growth is near +350 mV[197] depending on strain, metabolic state, and size of inoculum. Intense reducing conditions are established by the organism during logarithmic growth, probably due to the presence of low E_h substances such as ferredoxin. Final levels can reach below -400 mV.

pH

C. perfringens grows best at pH levels of between 6.0 and 7.0 with a range of between pH 5.0 and 9.0. The lower levels are reached in laboratory media containing readily fermentable carbohydrates. Clearly, some inactivation and/or injury of cells occurs during stomach passage due to the low pH levels (~ 2) encountered; however, the metabolic state of the cells is important because young, actively growing cultures are more susceptible to low pH than are stationary-phase cells.[57,90,289] The buffering capacity of proteins and the sheer bulk of the ingested food no doubt allows ent$^+$ cells to survive and subsequently sporulate in the small intestine. A recent report[289] indicated that acid exposure (pH 2.0, 30 minutes) actually enhances enterotoxin production (by four of five ent$^+$ strains).

Water Activity (a_w)

Typically the limiting a_w for growth of microorganisms depends on related conditions such as temperature, pH, and the solute used to adjust the a_w. Compared with *Staphylococcus aureus*, *C. perfringens* is not especially tolerant of low a_w. The lowest a_w supporting growth is between 0.97 and 0.95 using sucrose or sodium chloride (NaCl) respectively,[116] 0.93 with glycerol,[116] and 0.96 with glucose.[257] An awareness of limiting a_w would be important in newly introduced foods, such as intermediate-moisture foods.

Curing Salts

A level of 7% to 8% NaCl is required to prevent the growth of most strains of *C. perfringens*, although some inhibition occurs at levels of 5% to 6%.[39,68,168,213] In the case of sodium nitrite, levels of between 60 and 2560 ppm inhibit growth in filter-sterilized media, significantly less if nitrite is autoclaved with the test medium (due to the "Perigo effect").[199,208] The action of nitrite against *C. perfringens* has been reviewed by Craven.[39] Not surprisingly, lower concentrations of curing salts are required to inhibit growth when they are used together. For example, the inhibitory nitrite level decreased from 300 to 25 µg ml^{-1} when the amount of NaCl was increased from 3% to 6%.[213] Cured meats rarely are vehicles for outbreaks of illness due to this organism. For 1978 to 1992, only two such outbreaks (due to ham) were reported to the U.S. Centers for Disease Control and Prevention.

42.7.2 Spores and Sporulation

Effect of Temperature on Sporulation and Spore Inactivation

In complex culture media at 37° C, maximal levels of heat-resistant spores are attained usually within 7 hours;[123] in model food systems about twice as much time is required.[175] The higher the sporulation temperature, the greater the spore heat-resistance, an effect previously noted with *Bacillus* species.[133] For example, $D_{95°C}$ values (time for inactivation of 90% of the population at a specified temperature) of one ent$^+$ strain increased from 116 minutes (sporulation at 37° C) to 200 minutes (sporulation at 43° C).[61] Earlier reports[124] that *C. perfringens* cannot sporulate well at its optimal growth temperature (43° C to 45° C) have been disproven. Starch had been the principal carbohydrate in these investigations and α-amylase of most ent$^+$ strains was inhibited at the higher temperatures.[62] In the presence of raffinose (in place of starch), sporulation of many strains proceeds rapidly. This has been used to develop a rapid method for *in vitro* enterotoxin formation and purification.[97]

The ability of spores of *C. perfringens* to survive cooking is a principal contributing factor in its involvement in foodborne illness. Spores of type A strains vary widely in their heat-resistance. Significant differences in D-values exist between heat-sensitive (HS) and heat-resistant (HR) spores.[212] For example, in one study, the $D_{95° C}$ values for the HR group were between 17.6 and 63.0 minutes compared with between 1.3 and 2.8 minutes for the HS strains.[7] This explains, at least in part, the more frequent occurrence of the HR strains as the responsible agents in outbreaks; however, equally important, few HS strains produce CPE. Of 20 HR strains examined, only three were CPE-negative by reverse, passive latex agglutination (RPLA) (whether the CPE-negative strains had sporulated was not stated);[7] whereas of 17 HS strains, only one was RPLA-positive. A similar inverse relationship was noted between spore heat-resistance and lecithinase activity.[282]

In addition to genetic differences between strains, other factors that must be considered in comparing spore heat-resistance are the sporulation temperature and the medium in which the spores are produced and heated.[3,41,212,282] For example, heat resistance is greater in cooked meat than in water. It is important to use uniform conditions for comparing spore heat-resistance; this includes the use of clean spores (ie, free of their sporangia and vegetative cells). The D-values from a number of studies have been summarized elsewhere.[123]

As with spores of *Bacillus* species, spores of *C. perfringens* will germinate optimally only if they are mildly heated;[7] 75° C for 10 minutes is used typically. HR and HS spore strains respond differently to heat activation. In one study, about 50% of spores of the HS group grew without prior heat treatment compared with 0.13% to 3.6% for HR strains.[212]

Recovery of Spores from Thermal Injury

Spore injury and recovery is an important food safety issue. Unique among bacterial spore formers, *C. perfringens* (both HR and HS spore types) and *Clostridium botulinum*[198] spores can be recovered after thermal injury by inclusion of lysozyme in the plating medium.[32,54] Spore recovery rates of between 1% and 8% were achieved in the presence of 1 µg lysozyme ml^{-1} of plating medium compared with between 0.0001% and 0.0007% in its absence. Examples of the effect of lysozyme on D-values (time required for 90% reduction in viable counts) of *C. perfringens* spores are shown in Table 42–6. A similar though less dramatic phenomenon occurs using *C. perfringens* concentrated, supernatant fluid in place of lysozyme.[54] The responsible agent in this case was named initiation protein (IP). Both lysozyme and IP act at the level of germination and mimic the native spore-lytic enzyme[67] that is inacti-

Table 42–6 Effect of Lysozyme on Recovery of *C. perfringens* Spores[a]

Strain	*(ent[b])*	*Temperature (°C)*	*D-value[c]*	
			-Lysozyme	*+Lysozyme*
NCTC 8798	+	105	1.83	3.00
		120	0.01	0.52
NCTC 10240	+	90	7.66	21.66
		100	0.05	1.67
ACTC 3624	-	90	0.67	43.33
		100	0.01	3.00

[a] spores heated in 60 mM sodium phosphate buffer, pH 7.0

[b] enterotoxin-forming ability

[c] time (min) required for 90% reduction in viable spores

vated by the heating process.[263] The concern here is that lysozyme is a recognized food additive, and many foods naturally contain enzymes with lysozyme activity.

42.8 ISOLATION OF *C. PERFRINGENS* AND DETECTION OF ENTEROTOXIN

42.8.1 Criteria for Outbreaks

In addition to high numbers of viable cells in suspect foods, the presence of elevated fecal spore counts (those that survive heating at 75° C for 20 minutes) is also a diagnostic criterion. This was evaluated by assaying 66 stool specimens from nine outbreaks.[85] Using tryptose-sulfite-cycloserine (TSC) agar mean counts from outbreak stools were 1.4×10^6 cfu g^{-1} compared with 9.5×10^3 cfu g^{-1} from normal stools. Similar counts were obtained with sheep blood agar (without antibiotics). Only a minimal reduction in the spore count occurred following frozen storage of stools.

Current Public Health Laboratory criteria for establishing an outbreak of *C. perfringens* type A food poisoning include any one of the following:

- $> 10^6$ spores g^{-1} feces from ill individuals
- $> 10^5$ cells g^{-1} in incriminated food
- the presence of the same serotype in all individuals in an outbreak
- demonstration of the same serotype of *C. perfringens* in both contaminated food and feces

At least two problems have compromised three of these criteria: feces from many elderly individuals normally contain high numbers of *C. perfringens* spores and at least 25% of isolates do not react with available antisera. This has led to the view that the occurrence of outbreaks should be confirmed by direct detection of enterotoxin in feces of patients.

42.8.2 Cultural Methods for Detection

Detection

To avoid cold injury, food samples that require more than a 2-day storage should be mixed with a buffered glycerol salts solution to give a final concentration of 10% glycerol and be frozen as rapidly as possible to $< -50°$ C (eg, as on dry ice).[125]

The earlier efforts at development and use of plating media have been reviewed elsewhere.[122] The medium currently recommended in North America is TSC agar[87,88] in which *C. perfringens* appears as black colonies following the reduction of sulfite to sulfide. Its usefulness was confirmed by the work of Hauschild et al[95,96] who, at the request of the International Commission on Microbiological Specifications for Foods, evaluated many media proposed for enumeration of *C. perfringens*. The egg yolk–free variation (Table 42–7) is recommended because it is just as effective, simpler, and is a pour plate method. Incubation is at 37° C in anaerobic chambers under nitrogen or other anaerobic gas mixtures. As mentioned above, a count of $>10^5$ cells g^{-1} of food is sufficient to implicate suspect foods.

In the UK, surface-inoculated (horse) blood agar containing neomycin is used as well as TSC. The advantage of neomycin blood agar is that hemolysis by colonies is readily determined; however, neomycin can inhibit the recovery of some strains of this organism,[246] and neomycin-blood agar is not recommended for examining normal food samples in which the organism is present only in low numbers.

The simplicity and sensitivity of iron milk medium (IMM) should be mentioned. This most probable number (MPN) method uses pasteurized, whole milk supplemented with 2% iron powder.[249] Selection is based solely on the rapid growth of *C. perfringens* at 45° C and the typical "stormy fermentation" reaction within 18 hours, during which acid from lactose fermentation rapidly coagulates the milk and is followed by fractionation of the curd into a spongy mass. Confirmation is based on the acid phosphatase test.[1] Similar counts of *C. perfringens* were obtained from environment and food samples using TSC medium or the IMM method.[2,249] The IMM method for detection of *C. perfringens* in shell-

Table 42–7 Media for Enumeration and Confirmation of *C. perfringens* (Ingredient 100 ml^{-1})

Egg Yolk-Free Tryptose-Sulfite-Cycloserine Agar[a]	*(g)*	*Motility-Nitrate Medium*	*(g)*	*Lactose-Gelatin Medium*	*(g)*
Tryptose (Difco)	1.5	Beef extract	0.3	Tryptose (Difco)	1.5
Yeast extract	0.5	Peptone	0.5	Yeast extract	1.0
Soytone (Difco)	0.5	KNO_3	0.5		
Sodium metabisulfite	0.1	Na_2HPO_4	0.25	Na_2HPO_4	0.5
Ferric ammonium citrate	0.1	Galactose	0.5		
Agar	2.0	Glycerol	0.5	Gelatin[b]	12.0
pH adjusted to 7.6		pH adjusted to 7.3		pH adjusted to 7.5	
Cycloserine[c]	0.04	Agar[d]	0.3	Phenol red[e]	0.005

[a] Medium is the same as Shahidi Ferguson Perfringens (SFP) agar base (that contains no cycloserine) and is available commercially.

[b] Dissolved separately in 60 ml H_2O at 50 to 60° C.

[c] One ml of a 4% filter-sterilized solution added after pH adjustment and sterilization.

[d] Added after pH adjustment, then boiled to dissolve.

[e] Added as a solution after pH adjustment.

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fish has been adopted as first action by the Association of Official Analytical Chemists (AOAC) International.[1]

A method that uses lactose-sulfite (LS) broth and incubation at 46° C was proposed for rapid enumeration and identification of *C. perfringens* without further confirmatory tests.[182] It was found to be superior to TSC for enumeration of small numbers of the organism in a variety of foods.

Most recently a blood-free, egg yolk medium, BCP (*Bacillus cereus/C. perfringens*) was developed and shown to be equal or superior to TSC for unstressed (fresh, cooked-meat cultures) or injured (cold-shocked) cells respectively.[105] Counts from routine fecal samples or from infectious diarrhea were similar to those obtained on blood agar plus neomycin. The hydrogen peroxide degraders, catalase and pyruvate, each promoted the growth of several injured strains. Unfortunately, commercial egg yolk emulsion was not a satisfactory substitute for yolks prepared from fresh eggs.

Confirmatory Tests

The possible presence of other sulfite-reducing clostridia on TSC medium requires the confirmation of a representative number (about 10) of isolates. The recommended media for this purpose are tubes of motility-nitrate and lactose-gelatin media. Details on their use are described elsewhere.[81,125,215] Reported counts are obtained by multiplying the total count by the fraction of isolates that are confirmed as gram-positive, nonmotile rods and positive for nitrate reduction, lactose fermentation, and gelatin liquefaction. The use of TSC agar for enumeration and motility-nitrate medium together with lactose-gelatin medium for confirmation has been adopted as official first action by AOAC; however, the International Organization for Standardization and British Standard methods recommend the use of lactose-sulfite medium alone as a confirmatory test.[26,109]

In the UK in particular, the isolation of *C. perfringens* has been confirmed by streaking cultures on lactose–egg yolk medium to check for lecithinase activity.[104] This is seen as a zone of opalescence surrounding the colonies (Nagler reaction). Type A antitoxin is applied to half the plate to neutralize lecithinase produced by *C. perfringens*; occasional lecithinase-negative isolates would not be detected by this test.

A reversed CAMP test has been proposed for the presumptive identification of *C. perfringens*.[77] The procedure is based on the synergistic hemolysis (arrowhead-shaped) pattern produced on blood agar when *C. perfringens* is streaked at right angles to *Streptococcus agalactiae*. The procedure identified 97% of *C. perfringens* cultures tested with less than 1% false-negative reactions.[80]

A rapid (15 minutes) commercially available test (key iD strip) for presumptive isolated colonies is now available. Consisting of several paper-impregnated enzyme substrates, the test correctly identified all 73 *C. perfringens* strains tested and avoids the overnight incubation and requirement for type A antiserum.[25]

42.8.3 Serological Assays for Enterotoxin

The direct detection of CPE in outbreak stools is a useful technique for confirmation of *C. perfringens* as the etiological agent. Several serological assays for CPE have been developed including ELISA and RPLA, which have sensitivities of 2 to 4 ng g^{-1} feces.[16,123] Stools from healthy individuals contain undetectable levels of CPE[16] compared with outbreak stool samples, which contain >1μg g^{-1}.[13,16,17] A more recent ELISA method was described, with a detection limit of 1 pg ml^{-1} of purified CPE;[274] sensitivity in feces was not determined. A rapid ELISA (Tech Lab) and RPLA (Oxoid) are now commercially available. Instructions included with commercial CPE detection kit may be misleading by implying that a single sporulation medium will induce sporulation in all isolates whereas diarrheal strains often fail to sporulate *in vitro*.[119] An older serological assay method, counterimmunoelectrophoresis,[61,176] is used sometimes because of its simplicity although it is less sensitive.

Other serological methods for detecting CPE that have not been adopted widely include electroimmunodiffusion, reverse passive hemagglutination, fluorescent antibody, and single and double gel diffusion.[122]

42.8.4 Western Immunoblotting for Detection of Enterotoxin

Although ELISA and RPLA have proven very reliable, an occasional false-positive cannot be excluded.[13,190] In principle, Western immunoblots should provide a more specific CPE serologic assay because they can identify the 35-kDa CPE using antibodies prepared against purified CPE. The presumptive identification of a 48-kDa CPE-related protein as CPE[223] would also be avoided. Thus Western immunoblotting is the method of choice for verifying the enterotoxin-producing potential of clinical, food, and environmental isolates.[119] The drawbacks of the method remain the additional effort involved and the need to obtain at least low (up to 25%) levels of sporulation in cultures.

42.8.5 Gene Probe Methods for Detection of Enterotoxin-Producing (ent^+) Strains

Confirmation of the enterotoxin-producing ability of *C. perfringens* isolates by Western blotting or by serological methods depends on sporulation of the isolates. The difficulty of obtaining sporulation in laboratory media by this organism is well-known.[119,123] This led to development of methods for the direct detection of the *cpe* gene in vegetative cells of isolates. These detection assays include the use of PCR[228] and radioactive or digoxigenin (dig)-labelled gene probes.[9,119,268,277] Isolates identified as *cpe*-positive by such assays can only be regarded as potentially enterotoxigenic unless the ability of isolates to actually produce CPE is confirmed,[119] because it is not known whether isolates possessing the *cpe* gene also produce regulatory factors required for CPE expression.

Methods for detection of *C. perfringens* using a 16S rDNA-based PCR method[280] or 16S rRNA oligonucleotide probe method[217] have also been developed but do not specifically detect ent^+ strains.

42.8.6 Other Methods

Although tissue culture methods have been used previously to study the mode of action of CPE, the Vero cell line is also useful for assays of biological activity. Activity is measured as inhibition of plating efficiency[166,167] or killing of Vero cells.[146] Problems with reproducibility and nonspecific cytotoxic reactions were noted, however, when outbreak stools were used.[16] In the case of fecal samples, Vero cells were at least 10-fold less sensitive than ELISA or RPLA. The Vero cell assay is best used for mode of action studies or when biological activity of samples must be determined.

Many additional procedures have been used to study *C. perfringens*. These include bacteriocin typing,[281] plasmid profiling,[144,145,201] pyrolysis mass spectrometry,[238] immunomagnetic separation-ELISA,[43] dot-enzyme-linked immunosorbent assay (DOT-ELISA),[170] esterase electrophoretic polymorphism,[204] multilocus enzyme typing,[203] and impedance measurement.[250]

The sensitivity of *C. perfringens* to cold storage led to the development of methods to indicate previous growth of the organism in food. Lecithinase (alpha toxin) was proposed as an indicator because it is produced by the majority of strains.[83] To detect the enzyme in foods, a hemolysis indicator plate method was developed that requires at least 1.2×10^6 cfu g^{-1}

to have grown, although other workers found the minimum cell number to be about 10^4 cfu ml^{-1}.[58] The assay is dose-dependent above these thresholds. Other workers have found lecithinase activity to be time-, temperature-, and strain-dependent[180] and repressed in carbohydrate-supplemented media.[288] In addition, there have been occasional cases involving lecithinase-negative strains.[25,202] Thus the procedure is one of last resort, such as when patients' stools are unavailable or when the incriminated food has undergone prolonged cold storage. Nevertheless, the alpha toxin detection method has been adopted as official first action by AOAC for the examination of outbreak foods in which the presence of large numbers of vegetative cells are suspected but that may no longer be viable.

42.9 CONTROL

Unlike certain other agents of foodborne illness, it is not reasonable to expect *C. perfringens* to be absent from raw food products because the animal sources themselves harbor the organism. If present as spores, the organism can survive cooking, which effectively heat-activates the spores to promote germination when the product reaches a suitable temperature during cooling. The most practical way of preventing *C. perfringens* foodborne illness is by inhibiting the multiplication of the organism in food. Because the organism can multiply rapidly in protein foods, especially at 37° C to 45° C, food that has been cooked should either be kept above 60° C or cooled rapidly to below 7° C. Improper cooling after cooking has been identified as a principal cause of outbreaks in the US and UK.[27,211]

Cooking reduces the E_h of foods and thereby promotes anaerobic conditions; this is especially important in liquid foods and in rolled meats, where the contaminated outside surface is rolled into the middle. Other contributing factors that have been identified include inadequate hot holding and inadequate reheating of cooked, chilled foods. Reheating of cooked, chilled foods is important if the organism has had an opportunity to multiply because of previous temperature abuse. Such foods should be reheated to a minimal internal temperature of 75° C before serving to destroy vegetative cells. Certain public health agencies recommend holding cooked foods at or below 5° C (41° F) or above 60° C (140° F).[275] Such temperatures will effectively prevent the growth of *C. perfringens* in hazardous foods.

42.10 CONCLUDING REMARKS

Foodborne disease caused by *C. perfringens* continues to pose significant risk to human public health, especially in the aged or in individuals with underlying illnesses. Its widespread under-reporting impedes efforts at control and prevention. There must be greater awareness by public health agencies of its role in foodborne illness. The anaerobic conditions required to test outbreak foods for this organism are moderate in cost and effort.

The increasing demand and availability of minimally processed and *sous vide* foods present opportunities for growth of *C. perfringens* in products that have not been associated previously with it. This is particularly true given its broad temperature range and spore-forming ability.

Research efforts continue on determining the unique mode of action of the enterotoxin. As structure and/or function studies define specific epitopes of the toxin required for antigenicity, the possibility of a vaccine becomes possible. On another front, more rapid detection methods are needed that specifically identify ent^+ strains.

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43 *Escherichia coli*

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43.1 INTRODUCTION

The organism *Bacterium coli commune*, now known as *Escherichia coli*, was isolated by Escherich[123] from the stools of infants with enteritis. It was soon realized that *E. coli* was distributed widely in the intestines of healthy children and adults and other mammals. This commensal relationship, together with a lack of methods to differentiate *E. coli* strains, initially resulted in failure to recognize that some organisms had the potential to cause enteric disease. Differentiation of virulent and avirulent *E. coli* was achieved by the use of serological typing methods. These were first applied to study epidemic infantile enteritis[153] and subsequently developed by Kauffmann[208,209] to form a serological scheme that is the basis of the one used today. In the late 1940s, several *E. coli* strains associated with diarrheal disease were identified on the basis of their somatic O antigens,[43,147] and the division of the species into potentially pathogenic and commensal strains was established.

The importance of *E. coli* as a cause of diarrheal disease has been increasingly recognized over the past 25 years. Detailed comparison of the properties of diarrheagenic strains has resulted in the recognition of four categories of *E. coli* associated with disease. These are the enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and Vero cytotoxin-producing *E. coli* (VTEC). Other terms have been used to describe VTEC; these include Shiga-like toxin-producing *E. coli* (SLTEC) and more recently Shiga toxin-producing *E. coli* (STEC). The term enterohemorrhagic *E. coli* (EHEC) was introduced for those strains producing a bloody diarrhea;[228] therefore, EHEC are a subset of VTEC. EHEC possess virulence factors, in addition to Vero cytotoxin production, that are considered to be important in severe human disease. These properties are discussed in detail in Section 43.5.4. Strains of *E. coli* are identified as belonging to the above groups on the basis of their O and H (flagellar) antigens and the presence of specific virulence markers. There also may be features of clinical disease, epidemiology, and geographical distribution that are associated with particular categories. Further groups of *E. coli*, the enteroaggregative (EAggEC) and diffusely adherent strains (DAEC) have been linked epidemiologically in some studies with diarrhea.[121,329] Their virulence properties are defined less clearly than for the other groups, and the role of foodstuffs in their transmission is not established.

This chapter will consider the four major groups of *E. coli* that are associated with diarrheal disease in man. The newly emerging diarrheagenic groups will be included where information is available (Table 43–1).

Strains of *E. coli* have been associated with diarrheal disease in food-producing animals. These veterinary aspects have been comprehensively described recently.[165] Carriage of diarrheagenic *E. coli* by animals will be considered here only where there is evidence that strains harbored by animals pose a threat to human health either by contamination of food or by direct contact. In addition to diarrheal disease, strains of *E. coli* commonly cause urinary tract infections and a range of extra-intestinal diseases.[373] These infections are not foodborne and will not be considered here.

43.2 TAXONOMY AND TYPING

Castellani and Chalmers[54] first described the genus *Escherichia*, and the organism originally described by Escherich[123] became the type species *Escherichia coli*. The genus *Escherichia* is part of the tribe *Escherichiae* belonging to the family *Enterobacteriaceae* and contains five species. The other members of the genus are *E. hermanii*, *E. fergusonii*, and *E. vulneris*, all isolated from various human sources both intestinal and extra-intestinal, and *E. blattae*, only ever isolated from cockroaches. *E. coli* is the major species of the genus.

Escherichia coli are defined as oxidase-negative, Gram-negative rods ranging in size, 1-1.5 µm × 2-6 µm, usually motile by peritrichous flagella. They have an optimum growth temperature of 37° C but can grow over a wide temperature range from 15° C to 45° C. *E. coli* strains may also resist heating at temperatures of 55° C for 60 minutes and even 60° C for 15 minutes. Strains of *E. coli* grow well on most media forming smooth, circular, low convex colonies 2 to 3 mm in diameter.

E. coli are facultative anaerobes and ferment glucose with the production of acid and gas, although some strains are anaerogenic. The ability to ferment other carbohydrate sources is dependent on the conversion of relevant carbohydrate substrates to glucose or glucose derivatives. Lactose is fermented by most strains, but in some strains fermentation may be delayed or absent. Other carbohydrates usually fermented by *E. coli* include arabinose, glycerol, maltose, mannitol, rhamnose, and xylose. Fermentation of dulcitol, raffinose, salicin, and sucrose varies from strain to strain, and adonitol, cellobiose, and inositol are rarely fermented. Indole usually is produced and all strains are positive in the methyl red test and negative in the Voges-Proskauer test. Most strains do not hydrolyse urea or produce hydrogen sulphide in triple sugar iron (TSI) medium. Malonate is not used, phenylalanine is not deaminated, and gluconate not oxidized. Gelatin is not liquified, and almost all strains fail to grow in the presence of potassium cyanide (KCN). Most strains decarboxylate lysine and ornithine and are unable to use citrate as a sole carbon source (Simmon's citrate negative), whereas most strains of *E. coli* are able to use acetate as a sole carbon source. Strains also are capable of fermenting citrate in Christensen's medium, which is an important test to distinguish biochemically atypical *E. coli* from *Shigella*.[355]

As a result of the long association with disease, both in man and animals, many methods have been used to type and subdivide members of the *E. coli* species. These methods include colicin typing, biotyping, serotyping, phage typing, and, more recently, multilocus enzyme electrophoresis (MEE), pulsed field gel electrophoresis (PFGE), and restriction fragment length polymorphism (RFLP) analysis. Of all these methods, serotyping has been the most effective means of categorizing isolates of *E. coli* into

Table 43–1 Classes of Diarrheagenic *E. coli*

E. coli Class	*Toxins*	*Other Virulence Characteristics*	*Main O Serogroups*
Enteropathogenic (EPEC)	-	Bundle forming pili (BFP) Attaching and effacing lesions (intimin production)	55, 86, 111, 114, 119, 125, 126, 127, 128, 142, 158
Enterotoxigenic (ETEC)	Heat-stable, STI, STII, Heat-labile, LTI, LTII	Colonization factors associated with fimbriae or with the cell surface	6, 8, 15, 20, 25, 27, 63, 78, 128, 148, 153, 159, 167
Enteroinvasive (EIEC)	Enterotoxin (EIET) Cytotoxin	Invasion plasmid antigens (Ipa), 120 kDa outer membrane protein (VirG) necessary for cell-to-cell spread	28, 112, 124, 136, 143, 144, 152, 164, 167, 173
Vero cytotoxigenic (VTEC) or Shiga toxin-producing (STEC) (including enterohemorrhagic, EHEC)	Vero cytotoxins, VT1 and VT2 groups, Enterohaemolysin	Attaching and effacing lesions (intimin production)	5, 26, 91, 103, 111, 113, 128, 145, 157
Enteroaggregative (EAggEC)	Heat-stable, EAST 1, Heat-labile	Aggregative adherence fimbriae AAF/I, AAF/II	3, 44, 51, 77, 86, 92, 111, 126
Diffusely adherent (DAEC)	Not clearly defined	Adhesins, F1845, AIDA-I	1, 2, 21, 75

Note: The terms "pili" and "fimbriae" are used interchangeably.

closely related groups most frequently associated with particular virulence factors.

Serotyping is based on the identification of the following bacterial antigens. The somatic O antigens are composed of phospholipid-polysaccharide complexes. The specificity of an O antigen is determined by the terminal groups found in the repeating units of the polysaccharide chain. They are heat-stable (ST) antigens resistant to heating at 121° C. The capsular K antigen originally was used to describe the surface or capsular antigens that cause O inagglutinability. These acidic polysaccharide antigens originally were divided into three classes: A, B, and L. The A type K antigens are the most heat-stable, unaffected by heating to 100° C but their antigenicity is destroyed by heating at 121° C for 2½ hours. The existence of the B antigens has always been debatable, and Ørskov and others[281] recommended the withdrawal of this term for a K antigen. The L type antigens are heat-labile (LT) with their antigenicity destroyed by heating at 100° C for 1 hour. Only the A type K antigens are now considered to be important typing antigens, but they are associated mainly with pathogenic strains of *E. coli* from extraintestinal infections and not with those associated with diarrheal disease.

The flagellar H antigens are heat-labile protein antigens found in flagellin, the protein that constitutes the flagella found in motile *E. coli.* Strains usually are monophasic but rare diphasic strains have been reported.[299]

Kauffmann[208] devised a typing scheme incorporating all three types of antigen; he extended the scheme until, by 1947, it contained 20 O antigens, 55 K antigens, and 19 H antigens.[209] The scheme was adopted internationally and at present consists of O antigens, 1–173; K antigens, 1–103; and H antigens, 1–56. Currently, it is considered necessary only to determine the O and the H antigens to serotype strains of *E. coli* associated with diarrheal disease. The application of serotyping to isolates associated with diarrheal disease has shown clearly that distinct serogroups usually fall within the different classes of diarrheagenic *E. coli.*[127,238] The main O serogroups for each class are shown in Table 43–1. Some serogroups such as O111, O126, and O128 appear in more than one class. The use of bacteriophages for the epidemiological typing of *E. coli* strains was first promoted by Nicolle and colleagues.[274] Ahmed and others[7] developed a phage typing scheme for VTEC belonging to serogroup O157, and this scheme was later expanded.[214] The scheme uses 16 bacteriophages and now identifies 88 phage types (R. Khakhria, personal communication). In the United Kingdom (UK), the most common phage types found are 2, 21/28, 49, 1, 8, and 14,[2,303,386] whereas in Canada they are phage types 4, 8, 2, 1, and 31.[2,238]

43.3 CHARACTERISTICS OF THE DISEASE

Of all the *Enterobacteriaceae* found as part of the intestinal flora of man, *E. coli* is the predominant organism. *E. coli* are found in humans as part of the normal intestinal flora shortly after birth. Within a few hours, *E. coli* rapidly colonize the intestinal tract, becoming part of the normal flora within the large intestine. *E. coli* are ever present as part of the normal flora, a saprophytic commensal playing an important role in various physiological functions.[110]

43.3.1 Enteropathogenic *E. coli* (EPEC)

The disease caused by this group of *E. coli* is a watery diarrhea accompanied by vomiting and fever. The diarrhea is often self-limiting but EPEC can cause a protracted chronic enteritis leading to wasting and failure to thrive. EPEC are associated usually with infants and young children under the age of 3 years and are not routinely looked for in adults with diarrhea. EPEC were associated originally with large outbreaks of infantile enteritis in hospitals often with high mortality rates, but there have been few reports of outbreaks in the UK, Europe, or United States (US) since the mid 1970s. EPEC are still an important cause of diarrhea among children in the developing countries.[116,160,203,228]

43.3.2 Enteroinvasive *E. coli* (EIEC)

This group of *E. coli* produce a diarrhea that often clinically resembles bacillary diarrhea, the disease caused by *Shigellae.*[160,228,355] The diarrhea initially is acute and watery, accompanied by fever and abdominal cramps, progressing to the colonic phase where the stool becomes bloody and mucoid. Not all EIEC infections proceed to the colonic phase, so blood is not always detected in the stool. EIEC invade the colonic mucosa and multiply within the cells, spreading to adjacent cells after lysis of the infected cells. EIEC are biochemically atypical, often being anaerogenic, nonlactose fermenting, and lysine decarboxylase-negative, thus resembling *Shigellae.* Clinically, misidentification is not important but the accurate identification of the causative pathogen is epidemiologically important.

43.3.3 Enterotoxigenic *E. coli* (ETEC)

Disease caused by infection with ETEC is described as a watery diarrhea with abdominal cramps, fever, malaise, and vomiting. In its most severe form, infection with ETEC strains can produce a clinical picture resembling the diarrhea produced by *Vibrio cholerae*, that is "rice water stools." ETEC are a major cause of diarrhea in infants in the less-developed countries and also of travelers' diarrhea among those travelling from areas of temperate climate with good hygiene to areas, particularly in the tropics, with lower standards of hygiene.

43.3.4 Vero Cytotoxin-Producing (Shiga Toxin-Producing) *E. coli* (VTEC) (STEC)

VTEC were described first by Konowalchuk and colleagues in 1977,[220] but it was not until 1982 that they were recognized as significant causes of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS).[207,309] Symptoms of HC often begin with abdominal pain and watery diarrhea, followed by bloody diarrhea usually without fever. The diarrhea, either bloody or nonbloody, may be followed by the development of HUS. This is characterized by microangiopathic hemolytic anemia and thrombocytopaenia, followed by acute renal failure. HUS occurs in all age groups but is most common in young children. The spectrum of disease caused by VTEC also includes thrombotic thrombocytopenic purpura (TTP).[206] With TTP, the clinical features of HUS are accompanied by fever and a low platelet count associated with formation of thrombi that give rise to severe neurological disorders.

43.3.5 Enteroaggregative and Diffusely Adherent *E. coli* (EAggEC and DAEC)

Enteroaggregative *E. coli* have been associated with persistent diarrhea in developing countries.[329] This diarrhea lasts for more than 14 days and usually is watery with vomiting, dehydration, and abdominal pain in some patients. Bloody diarrhea and fever have been described in children infected with EAggEC.[30,87] Diarrhea associated with diffusely adherent *E. coli* is characterized by mucus-containing watery stools with some fever and vomiting.[121]

43.4 EPIDEMIOLOGY OF *E. COLI* CAUSING DIARRHEAL DISEASE

There are significant differences in the incidence of outbreaks and sporadic cases of infection caused by the major groups of diarrheagenic *E. coli*. Changes in the epidemiology of infection due to some types of strains have been observed over the years. Different factors are of importance in developed countries compared with the developing world.

43.4.1 Epidemiology of EPEC

EPEC Infection in Developed Countries

Historically, these strains comprised the first group of *E. coli* for which epidemiological studies were carried out by applying a serotyping scheme. In contrast to the "summer diarrhea" that had been the annual scourge of infants in Europe and the US until the 1930s, outbreaks of infantile enteritis were observed with increasing frequency during the 1940s, principally during the winter. Investigation of nursery outbreaks in London and Aberdeen, Scotland, implicated *E. coli* of serogroups O55 and O111 as the causative agents.[43,147,366] Many epidemics of EPEC infection were reported in Europe and North America in the 1950s and continued in Britain until the early 1970s. Serotypes isolated from infected infants included O114:H2, O119:H6, O127:H6, O128:H2, and O142:H6.[160] An important feature of these outbreaks was a high attack rate accompanied by high mortality, which could exceed 50%. The highest rates of infection were in babies less than 6 months of age, particularly those that were bottle-fed.

Studies of outbreaks in Aberdeen and Dublin[147,192,366] indicated that infection was spread within the nursery after admission of infected infants from the community. Strains of EPEC excreted by infants caused widespread contamination of their physical environment and carers.[314] Transmission occurred by the fecal-oral route mediated by contaminated formula feeds and feeding equipment. Dissemination of EPEC on a global scale was indicated by retrospective studies of outbreaks in England, Scotland, Northern Ireland, Eire, Canada, Indonesia, and the US.[162] It appeared that a single EPEC clone of *E. coli* O142 was responsible for disease in all these countries.

Since the 1970s, there has been a marked change in the epidemiology of EPEC in developed countries. Outbreaks of infantile diarrhea have become rare in Britain and the US. This has been attributed to improvements in hospital hygiene, improved preparation and formulation of milk feeds, and better nursery management to avoid cross-infection. Sporadic cases of EPEC infection in children continue to be reported, with a peak in the summer months. There were 387 reports of diarrhea associated with EPEC in England and Wales in 1994 in children less than 3 years old (Communicable Disease Surveillance Centre, unpublished). There remains the potential for severe disease, exemplified by an outbreak caused by *E. coli* O114 in a daycare center in the US in which there was one death and secondary spread of infection.[41]

The contribution of EPEC to diarrheal disease in adults in the developed world is difficult to evaluate because most laboratories do not examine stools from patients over 3 years of age for these organisms. A few outbreaks have been reported, and in some of these a link with food or beverages has been established epidemiologically or by isolation of the organism from patients and from the incriminated food. In a waterborne outbreak in the US, *E. coli* O111 was isolated from stools and from well water that had been contaminated by sewage.[336] Multiple EPEC serogroups were isolated from a water supply that caused an outbreak in Sweden (quoted by Doyle & Padhye, 1989).[107] Outbreaks linked to food have been reported in Britain.[399] A large outbreak of diarrhea caused by *E. coli* O111 affected 650 children and adults in Finland and spread to 137 household contacts, but the source of the organism remained unknown.[401]

EPEC Infection in Developing Countries

In the developing world, or where standards of hygiene are poor, EPEC strains remain a major cause of diarrhea in infants. Outbreaks occur in institutions and the general community, together with sporadic infections.[85,116,154,203,229] The peak incidence of disease occurs in the warm season. Breastfeeding, which may continue longer in developing areas, provides a protective effect against infection.[349] Infants generally fall ill in the months following the start of weaning, probably because of fecal contamination of weaning foods by the water supply. Individuals may suffer several diarrheal episodes in the first three years of life.[85]

Studies on the epidemiology of EPEC have indicated that the reservoir of these strains is the gastrointestinal tract of man and that there is no evidence for zoonotic infections.

43.4.2 Epidemiology of ETEC

ETEC Infection in Developed Countries

Surveys of sporadic diarrhea in the US, Canada, Sweden, and Britain have indicated that ETEC are not an important cause of disease in developed countries in children or adults;[160] however, ETEC have caused outbreaks of infantile enteritis in hospitals in England, Scotland, and the US.[161,257,318,321] The epidemic strains isolated from these outbreaks included serogroups O6, O78, and O159. The sources and routes of transmission were unclear, although cross-infection was very important. In an outbreak that continued for nine months in the US,[321] there was extensive environmental contamination with the causative *E. coli* O78. The organism was recovered from milk feeds but not from unopened containers of formula. Contamination of food or water by ETEC may occasionally result in outbreaks of infection in the community. More than 200 staff and visitors at a US national park fell ill after contamination of the water supply with raw sewage. *E. coli* O6 was isolated from patients and drinking water.[315] Contaminated well water was associated with outbreaks in Japan.[223] Outbreaks of infection linked to foods have been reported in Japan,[223] England,[311] and the US.[55,240] Implicated vehicles of infection included contaminated turkey mayonnaise, imported French cheese, and salad vegetables. Individual food handlers with diarrhea have been implicated as the cause of some outbreaks.[381]

ETEC Infection in Developing Countries

Strains of ETEC are an important cause of diarrhea in all age groups in regions of poor hygiene, particularly in the tropics.[160] Rates of infection are highest in infants up to 2 years old and decline in older children and adults, indicating progressive development of immunity.[36,231] Infants may suffer two or three clinically significant episodes of diarrhea per year due to ETEC, particularly around the time of weaning.[33] Together with diarrhea of other etiology, this contributes to the development of clinical malnutrition, which in turn may reduce significantly the dose of ETEC required to cause diarrhea. Infections caused by ETEC reach a peak in the warm season. Infants are vulnerable to contaminated weaning foods but all may be exposed to water supplies contaminated by latrines or as a result of multiple uses.[34,36] Water stored in homes may become a source of ETEC that may also be transmitted on contaminated hands.[119] Studies in developing countries have shown that ETEC associated with human disease can be recovered from rectal swabs of healthy domesticated animals, including cattle and pigs.[36,120] This suggests that livestock may represent a further source of environmental contamination.

Travelers' Diarrhea

Travelers from temperate countries where standards of sanitation and hygiene are high are at risk of infection when visiting regions where ETEC are endemic. The relationship between ETEC and travelers' diarrhea was first demonstrated in studies of British troops arriving in southern Arabia.[320] Later studies on US and British military personnel deployed to Vietnam, the Philippines, Thailand, and the Arabian Gulf have supported this finding.[112,115,193,414] In Mexico, several investigations of travelers' diarrhea showed attack rates between 29% and 48%, with ETEC detected in about 50% of those ill.[113,155,251] In one Mexican study, consumption of salads containing raw vegetables was linked epidemiologically to infection.[251] This last study indicated that asymptomatic carriage of ETEC by travelers is uncommon.

In addition to the more obvious epidemic areas, travelers have acquired ETEC in Mediterranean countries. Surveys in Sweden and Britain revealed that 11% of travelers who developed diarrhea while abroad or shortly after return suffered from ETEC infection. There have been a number of incidents of ETEC infection on cruise ships, sometimes involving successive cruises by the same vessel. Hobbs et al.[186] reported the isolation of *E. coli* O27 from 55%, 61%, and 20% of diarrheal cases in three cruises by the same ship. These infections probably were foodborne. Microbiological and epidemiological evidence implicated the water supply on another ship as the source of infections, which were caused by ETEC of several different serogroups.[280]

43.4.3 Epidemiology of EIEC

Strains of EIEC were identified first in Allied troops suffering from dysentery in the Mediterranean during World War II.[128] The association of these organisms with an outbreak of disease was established when school children in Britain became infected with *E. coli* O124 after consumption of a midday meal.[187] Although the outbreak was thought to be foodborne, the organism was not isolated from a food item.

There have been several outbreaks of EIEC infection throughout the world. Settings have included hospitals and institutions in Britain, Australia, Czechoslovakia, and the US.[177,271,310,317,319] Both adults and children have been affected. Outbreaks of infection probably are foodborne, although the association usually has been epidemiological. However, in one investigation, a high level of *E. coli* O124 contamination of imported French cheese was linked directly with a large outbreak of infection in the US.[242] The source of contamination appeared to be improperly filtered river water used to clean the cheese-making equipment. Transmission of infection from person-to-person was identified during an outbreak of diarrhea associated with *E. coli* O124 in the US.[177,178]

Sporadic cases of EIEC infection appear to be rare in the developed world, although incidence may be underestimated because of the difficulty most clinical laboratories would have in identifying the organism. Like EPEC and ETEC strains, EIEC are endemic in many developing countries.[118,379] Children between 3 and 5 years of age are most at risk,[380] and the organism can cause travelers' diarrhea.[380,404] The reservoir of EIEC appears to be the gut of infected humans.[107] This conclusion has been based on the similarity of the organism to *Shigella* and a lack of evidence for animal carriage or for specific association with foods of animal origin.

43.4.4 Epidemiology of VTEC

In contrast to infections with EPEC, ETEC, and EIEC, diarrheal disease caused by VTEC has been recognized as an emerging problem, particularly in North America, Europe, and Japan. Surveys of nonselected diarrheal stools in North America and the UK indicated that between 0.1% and 2.7% contained O157 VTEC.[156,158,241,323] There have been few investigations of the incidence of VTEC other than O157, largely because of the lack of simple tests for the primary isolation of these organisms. Surveys of diarrheal stools in Canada,[283] Germany,[164] and Belgium[293] indicated that 0.7%, 6.6%, and 1% contained non-O157 VTEC, respectively; the corresponding findings for O157 VTEC were 2.5%, 2.7%, and 0.2%.

Several studies have established that O157 VTEC are a major cause of HC. In North America and the UK, these organisms have been isolated from between 15% and 70% of bloody stools.[156] Since Karmali et al.[207] first established the association between VTEC and HUS, many studies in North America and Europe[51] have shown that O157 VTEC are more commonly isolated from patients with HUS than are VTEC of other serogroups. Rates of isolation of O157 VTEC have ranged from 19% to more than 60% (summarized from Griffin and Tauxe[156]) although evaluation of the data is difficult because studies have varied in size and techniques used to identify VTEC strains. In England and Wales, HUS develops in about 10% of patients infected with O157 VTEC.[386] HUS has been recognized for many years as the most common cause of acute renal failure among infants and children in Argentina and Chile.[156,206] Evidence of VTEC infection in cases of HUS has been reported in Chile, Uruguay, and Argentina.[83,239] Currently, the annual incidence of HUS in children under 5 years of age in Argentina is 7.8 per 100,000,[423] with evidence of VTEC infection in about 70% of cases. O157 VTEC are most commonly associated with HUS patients with bloody or nonbloody diarrhea.

In a number of studies in Canada and the US, O157 VTEC strains were the second or third most frequently isolated enteropathogen and were more common than *Shigella.*[156] In Canada, the province of Alberta had the highest isolation rate of 15 per 100,000 population.[4] Strains of O157 VTEC are more frequently isolated in the northern and western US than in the South.[158]

The incidence of infections caused by O157 VTEC in England and Wales has been monitored by the Laboratory of Enteric Pathogens since 1983.[4,385,386] There has been a significant increase in the number of isolates of O157 VTEC and a rise in outbreaks caused by this organism in Britain.[352,360,402] For the period 1990 to 1996, the average annual isolation rate of O157 VTEC in England and Wales was less than 1.5 per 100,000, whereas in Scotland the average rate for this period was greater than 5 per 100,000. There is considerable geographical variation in the rates of isolation. In England for 1992 to 1996, the highest rates of O157 VTEC infection have been recorded by Northern, Trent, East Anglia, South West, and North West Health Authorities.[386] The lowest rates were in the Thames regions. Some of the variation seen in regional incidence in England and Wales may be the result of significant differences in ascertainment. In Scotland the highest rates between 1990 and 1994 were seen in Grampian and the Borders. Since then, rates of between 4 and 10 per 100,000 have been recorded in several Health Board areas, and in 1996 the Borders had a rate of 21.7 per 100,000 without any large reported outbreaks.[303] The increases probably result from improved isolation techniques and better ascertainment as well as a true increase in infections caused by O157 VTEC.

Surveillance of O157 VTEC infections in sporadic cases and outbreaks has shown that the age range of affected individuals in England and Wales is very wide—from less than 6 months to more than 80 years, with a peak in the 1 to 4 year age group.[385,386] Mortality rates are highest in young children and the elderly as seen in several outbreaks (Table 43–2). The organism is not an opportunistic pathogen and has caused fatal illness in previously healthy individuals. In contrast, investigations of contacts of patients has shown that some people can harbor O157 VTEC asymptomatically.[156,324] Studies in the US and Britain have shown a marked seasonality with peak incidence of infections in mid to late summer.[156,385,386]

Reported outbreaks of infection caused by VTEC have been predominantly associated with strains of serogroup O157. Table 43–2 lists examples of such outbreaks chosen to represent different countries, settings, food vehicles, and other modes of transmission. Community outbreaks have been recognized often by the appearance of a cluster of HUS cases; however, the proportion of patients within an outbreak who develop HUS is extremely variable[156] and is not a reliable outbreak marker. The first reported community outbreaks of infection with O157 VTEC occurred in the US in 1982 in Oregon and Michigan.[309] They were linked to consumption of ground beef sandwiches from the same restaurant chain, and O157 VTEC were isolated from frozen beef patties.[409] A very large outbreak (Table 43–2) occurred in the western US in 1993 and involved undercooked hamburgers from multiple outlets of a restaurant chain.[20,157] There was a significant number of secondary cases. Identification and withdrawal of the contaminated product were considered to have limited the scale of the outbreak. Although beefburgers from a restaurant chain were linked epidemiologically to O157 VTEC infection in Britain in 1991, the first isolation of O157 VTEC from a beefburger was associated with a small community outbreak in Wales.[421]

Some very large and serious outbreaks of O157 VTEC infection occurred in 1996. In Japan in 1996, more than 9,000 cases were reported in several different prefectures between the end of May and September with more than 5,000 people ill in Sakai City in July.[267,406,423] There were 16 outbreaks between May and November 1996,[252] in which school children were predominantly affected; it was suggested that school meals were the vehicle of infection but the specific sources were not identified. In Central Scotland, an outbreak occurred in November and December 1996, in which 501 cases were identified and 21 elderly people died.[8,291] The infections were associated with consumption of meat originating from a butcher and the O157 VTEC "outbreak"

Table 43–2 Examples of Outbreaks Caused by O157 VTEC Illustrating Possible Modes of Transmission

Year	*Location/Setting*	*Cases*	*Fatal*	*Transmission/Vehicle of Infection*	*Reference*
1982	Michigan, Oregon, US, Community	26		Hamburgers	309
1985	East Anglia, England, Community	49	1	? Handling raw vegetables	261
1985	Canada, Nursing home	73 (12 HUS)	17	? Sandwiches, person-to-person	52
1989	Missouri, US, Community	243 (2 HUS)	4	? Water	375
1990	Japan, Kindergarten	319	2	? Well water	222
1991	Preston, England, Fast food restaurant	23 (3 HUS)		? Beefburgers	385
1992/3	Western US, Fast food restaurants	732 (55 HUS)	4	Hamburgers	157
1992	Stockport, England, Hospital	14	3	Person-to-person	386
1993	Sheffield, England, Farm dairy/Community	7 (3 HUS)		Raw milk	69
1993	Gwent, Wales, Community	17 (1 HUS)		Butcher meat	421
1993	London, England, Community	7 (3 HUS)	1	Paddling pool/person-to-person	183
1994	Leicestershire, England, Visit to community farm	7 (4 HUS)		Animal contact	354
1994	Scotland, Community	100 (9 HUS)	1	Pasteurized milk	397
1996	Sakai City, Japan, Schools, Community	5,727 (>100 HUS)	3	? White radish sprouts	267,423
1996	Central Scotland, Community, Nursing home	501 (27 HUS)	21	Cooked meat, gravy	8, 291, 337, 338
1996	US, Canada, Community	66 (12 HUS)	1	Unpasteurized apple juice	57, 58

strain was isolated from gravy and also from a cooked beef joint.[337,338]

A range of food products of animal origin has been implicated or proven to be the vehicles of infection in outbreaks. In England, a small outbreak was caused by consumption of raw milk, and in Scotland at least 100 individuals became ill after drinking milk that became contaminated after pasteurization (Table 43–2). Some outbreaks have been linked epidemiologically to food vehicles such as sandwich fillings, cooked meats, and raw vegetables.[143,248,261,370] It is likely that the source of the organism in these cases was cross-contamination from raw meat or the exposure of raw vegetables to water contaminated with cattle feces. Occasional waterborne outbreaks of infection have been reported; one large incident in the US followed probable contamination of a municipal water supply after pipe fractures (Table 43–2).[375] The role of foods in VTEC infections will be discussed in Section 43.7.2.

Outbreaks of O157 VTEC infection in hospitals, nursing homes, and daycare centers have sometimes been associated with a high incidence of HUS and high mortality (Table 43–2).[52,385] In these institutional settings, person-to-person spread by the fecal-oral route has been of great significance after the initial cases. Assessment of the infectious dose of O157 VTEC from foodborne outbreaks has indicated that fewer than 100 organisms may cause disease.[4,421] Person-to-person spread is likely to be a factor in the large number of small family outbreaks of O157 VTEC infection that occur each year. A further mode of transmission of infection is by direct or indirect contact with animals, particularly cattle. In England, children and adults who visited a community farm became ill with O157 VTEC.[354] They had contact with cattle, goats, and other animals and had eaten a picnic meal without washing their hands. Identical O157 VTEC strains were recovered from patients, cattle, and goats. The role of animal carriage as a source of infection will be discussed later in this chapter.

Although some strains of VTEC other than O157 are human pathogens, they have been responsible for relatively few outbreaks of diarrhea or bloody diarrhea (Table 43–3). In Japan, a VTEC strain of serotype O145:H- caused an outbreak of diarrhea in a school,[199] and further Japanese outbreaks have involved strains of serotypes O111:H- and O104:H21.[222] Reports from Italy[50] and Australia[49,290] have described community outbreaks of HUS caused by VTEC of serogroup O111. In the Australian outbreak, there were over 200 cases; 22 children developed HUS and 1 died.[423] The source of the latter outbreak was traced to mettwurst, a fermented sausage. An outbreak in Montana resulted from contamination of pasteurized milk with VTEC of serotype O104:H21.[56]

43.4.5 Epidemiology of Newer *E. coli* Groups: EAggEC and DAEC

Studies on infants and children in developing countries have disagreed about the association between EAggEC and diarrhea. Gomes and colleagues[154] and Baqui et al.[16] did not find an association, whereas studies in India, Mexico, and Brazil have found a significant association of EAggEC with persistent diarrhea in children.[29,30,87,405] Strains of EAggEC have been isolated from travelers returning to Britain from abroad.[45,345] Cases of sporadic diarrhea in Britain have been linked to EAggEC, and the strains included a proportion that belonged to serotypes O44:H18, O111ab:H25, and O126:H27.[343,348] These three serotypes were recognized previously as EPEC serotypes based on epidemiological studies. Strains of serotype O44:H18 also have caused outbreaks of infection among young children and the elderly.[343,362] In 1994 several outbreaks of gastroenteritis in the UK were associated with EAggEC *E. coli* and contaminated foods were thought to be the source of the infections.[359] A preliminary study of infectious intestinal disease in England has indicated that EAggEC were found more frequently in cases than controls.[313]

Epidemiological studies of DAEC have been carried out in developing countries. Whereas some reports have found a significant association between these strains and diarrheal disease,[16,150,244] the majority of studies have failed to do so.[30,87,233] A more recent study indicated that the risk of infection with DAEC was greater in children between 4 and 5 years of age than in infants.[230] The incidence of DAEC in England is being studied as part of a study on infectious intestinal disease, but data are not yet available.

43.5 PATHOGENESIS MECHANISMS

43.5.1 Enteropathogenic *E. coli*

The adherence of EPEC to the intestinal mucosa, rather than toxin production, is the important mechanism in their pathogenesis. Polotsky et al.[295] observed histopathological lesions in rabbit ligated

Table 43–3 Examples of Outbreaks Caused by Non-0157 VTEC

Year	*Location/Setting*	*Cases*	*Fatal*	*Serotype*	*Transmission/Vehicle of Infection*	*Reference*
1984	Tokyo, Elementary school	100		0145:H–	Unknown	199
1986	Japan, Orphanage	22	1	0111:H–	Unknown	222
1991	Japan, Elementary school	234		0111:H–	Unknown	222
1991	Tokyo, Elementary school	89		0?:H19	Unknown	222
1992	Italy, Community	9 (all HUS)		0111:H–	Unknown	50
1992	France, Community	10 (all HUS)		0111	Mainly person-to-person	40
1994	Montana, US, Community	19		0104:H21	Pasteurized milk	56
1994/95	South Australia, Community	>200 (22 HUS, 4TTP)	1	0111:H–	Mettwurst	49, 290, 423
1995	Spain, School	13		0111:H–	? Drinking water	37

intestinal loops infected with classical EPEC strains, and similar lesions were seen also with intestinal biopsy material from infants with diarrhea.[316,396] This attaching and effacing (AE) lesion was characterized in detail by electron microscopy and shown to involve close attachment of the EPEC to the epithelial cells and destruction of the brush border.[218,259] Another advance in the study of EPEC pathogenesis came from the observation that "classical" EPEC isolated from outbreaks adhered to HEp-2 cells.[84] The bacteria formed clusters, later termed the localized pattern of attachment, whereas other types of *E. coli* and those from the normal flora rarely showed this adhesion. A plasmid, termed the EPEC adherence factor (EAF) plasmid, is necessary for the full expression of adhesion to HEp-2 cells.[13] Volunteer studies supported the role of this plasmid in EPEC pathogenesis.[232] Nine of ten subjects fed the wild-type EPEC strain had clinical diarrhea compared with only two of nine people receiving the plasmid-cured derivative. Chromosomal virulence factors are required in addition to the EAF plasmid.

Recent morphological and genetic studies have identified distinguishable stages of the adherence process.[411] The first step, resulting in localized adherence, is thought to be mediated by bundle-forming pili, the production of which is determined by the EAF plasmid.[149] There is then an alteration of the host cytoskeletal structure, including effacement of the surface microvilli, followed by the appearance of a pedestal-like structure on which the bacteria are localized. Intimate attachment to the epithelial cells requires a bacterial outer-membrane protein, termed intimin, encoded by the chromosomal *eae*A gene. Host cell responses following contact with EPEC include protein phosphorylation, increase in calcium levels, and rearrangement of cytoskeletal components under the adherent bacteria. The effect of these changes is that there is a significant reduction in the absorptive capacity of the brush border and a stimulation of intestinal secretion.

43.5.2 Enterotoxigenic *E. coli*

The pathogenesis of ETEC requires colonization of the small intestine and production of one or both of the two types of enterotoxin, LT and ST. The mechanisms of action of these toxins have been reviewed in detail.[263,350] Two types of LT enterotoxin are now recognized, designated LTI and LTII. LTI is related closely in its structure, function, and antigenicity to cholera toxin (CT) produced by strains of *V. cholerae*.[166,189] The structure comprises one polypeptide A subunit and five polypeptide B subunits, with molecular weights of approximately 30 kDa and 11.5 kDa respectively. The B subunits form a highly stable structure held together by hydrogen bonding and salt bridges. The A subunit is synthesized as a single polypeptide, but is split by proteolysis into a large enzymatically active peptide (A_1) and a small peptide (A_2) that connects the A subunit to the B pentamer. The A_1 and A_2 peptides are joined by a single disulphide bond that remains intact until the toxin enters a cell.

Each of the B subunits binds to the lactose-containing oligosaccharide portion of the monosialoganglioside GM1 in cell membranes.[159] Binding to the monosialoganglioside, GM2 and asialo GM1 has also been demonstrated.[190] Each B pentamer of LTI can bind five GM1 molecules; following this, the toxin is internalized. LTI and CT possess the same enzymatic function of adenosine-diphospho-ribosyl transferase activity in the A_1 fragment of the A subunit. Adenosine diphosphate (ADP)-ribose is transferred from nicotinamide adenine dinucleotide to a major specific target in the cell. This ADP-ribosylation occurs in the brush border membrane of the intestinal epithelial cell and results in activation of adenylate cyclase and a subsequent rise in the level of cyclic adenosine 5^1–monophosphate (cAMP). This stimulation of cAMP leads to electrolyte disturbances with increased secretion of chloride from crypt cells and impaired absorption of sodium and chloride by cells at the tips of the villi.[133,134] Water follows the electrolytes with a resultant profuse watery diarrhea.

LTI can be further subdivided into LTIh (human) and LTIp (porcine).[191] These toxins are closely related but can be distinguished immunologically: LTII differs from LTI in that it is not neutralized by antisera to CT or LTI. The structure of LTII is similar to that of LTI,[188] but LTII binds to the disialoganglioside receptors GD1a and GD1b and not GM1.[142] The enzymatic activity of LTII is the same as that of LTI, but production of LTII by *E. coli* is about 1% of the level of LTI. Like LTI, antigenic variation has also been observed with LTII, and subtypes LTIIa and LTIIb have been reported.[166] The properties of LTII have been characterized, but the role of this toxin in human disease has not yet been established. In contrast to the chromosomal CT genes, structural genes for LTI are located on plasmids and sequence analysis has shown >95% homology between LTIp and LTIh. There is approximately 70% homology between LTI and CT.[425] The structural genes for LTII are chromosomal, and the genes for the A subunits of LTIIa and LTIa have 57% nucleotide sequence similarity, but the B subunit genes for the two toxins lack significant homology.[292]

There are two major types of ST toxins: STI (or STa) and STII (or STb). These toxins are unrelated apart from their heat stability, secretion from the cell, and ability to cause diarrhea.[166] STI has a molecular weight of about 2000 and is resistant to 100° C for 15 minutes. The molecule is acid-resistant, susceptible to alkaline pH, and inactivated by reducing and oxidizing agents that disrupt disulphide bonds.[312] The STI toxins have been subdivided into STIa and STIb; they share antigenic determinants and biologically active sequences that are at the C-terminal end. The differences between STIa and STIb are at the N-terminal end, with identical central sequences of 11 amino acids.[371] STI binds to a specific receptor, reported to be a transmembrane guanylyl cyclase, located on brush borders of intestinal epithelial cells.[97,98,185] Binding of STI is maximal in villus preparations and decreases from villus to crypt.

The action of STI is to activate particulate guanylate cyclase in the brush border of the intestinal epithelial cells in the jejunum and ileum, leading to elevated levels of cyclic guanosine monophosphate (GMP).[132] The net effect is the inhibition of sodium-coupled chloride absorption in villus tips as well as stimulation of chloride secretion in crypt cells, leading to excessive fluid accumulation in the gut lumen.[136] The action of STI is very rapid but has a short duration. STI receptors are more widely distributed in the colon than in the ileum, and diarrhea caused by ST-producing *E. coli* represents the combined effects of fluid secretion in the small intestine and impaired absorption in the colon.

STII is a peptide comprising 48 amino acids but, unlike LT or STI, this toxin does not alter intracellular levels of either cAMP or cGMP. The mechanism of action is not known at present, although it has been proposed that STII acts by opening a receptor-operated calcium channel in the plasma membrane.[111] STII is produced by ETEC predominantly of porcine origin, usually in combination with other enterotoxins, particularly LTI.[53,166] STII, like STI, acts rapidly and for a moderate period with maximum fluid accumulation after 3 hours.

The genes for STI and STII are located on plasmids, and in the case of STIa and STII, the genes are carried on transposons.[226,369] DNA sequence analysis showed that STIa and STIb genes encoded products of 18 and 19 amino acids, respectively. STIa is synthesized as a 72 amino acid precursor that consists of a 19 amino acid signal peptide, a 35 amino acid pro sequence, and the 18 amino acid STIa. The DNA sequence for STII has also been reported: the structural gene encodes a mature protein of 48 amino acids and a signal peptide of 23 amino acids.[226]

Studies of enteritis in pigs first demonstrated that enterotoxin production was not sufficient to enable ETEC to cause diarrhea,[364] and this was shown later for strains of human origin. Adherence to the mucosal surface of the intestinal epithelial cells was the first essential step in ETEC pathogenesis. Most ETEC adhesins are nonflagellar, hair-like, rigid filamentous structures termed fimbriae or pili. ETEC fimbriae are host-specific and particular fimbriae are found exclusively on strains of either human, bovine, or porcine origin.[93,94]

Colonization factor antigen I (CFA/I) was the first adhesin on human ETEC to be characterized, and its role in disease was demonstrated in volunteer experiments.[125,126] CFA/I is a single antigen assembled into a rod-like fimbrial structure of 6–7 nm in diameter. In contrast, CFA/II comprises three antigenically distinct proteins—coli surface-associated antigens CS1, CS2, and CS3.[86,367] CS1 and CS2 are fimbrial antigens, while CS3 has a fibrillar structure; strains may produce CS1 and CS3, CS2 and CS3, or CS3 alone. CFA/IV comprises three antigenic components, CS4, CS5, and CS6. Several other colonization factors or putative colonization factors (PCF) produced by human ETEC have now been identified.[94,245,368]

Fimbriae are homopolymers composed of several hundred identical protein subunits, but in some cases there are minor proteins that are the actual adhesins. CFA/I fimbriae consist of a single type of subunit, of which only the tip-located subunit is accessible to the receptor.[47] The fimbrial adhesins of ETEC bind to glycoconjugates, glycoproteins, and glycolipids. This adhesin-receptor interaction occurs in a lectin-like fashion with the receptors located in mucus, epithelial membranes, and basement membranes.[93] The host specificity of ETEC results from the diversity of these glycoconjugates and their host distribution. Although most adhesins of ETEC have a fimbrial morphology, others such as CS6 are nonfimbrial. The adhesins in ETEC of human origin are usually encoded by plasmids.[368]

43.5.3 Enteroinvasive *E. coli*

Enteroinvasive *E. coli*, like *Shigella*, cause dysentery by invading intestinal epithelial cells. The invasion process includes several stages: entry of the bacteria into epithelial cells, intracellular growth, intracellular movement, and cell-to-cell spread. The result of these events is death of the host cell. The cellular and molecular basis of invasion and the genetic control have been reviewed.[172]

The entry of EIEC *E. coli* into mammalian cells requires energy production by bacteria and the host cell.[170,171] There is recognition of host-cell receptors, and entry is followed by reorganization of the cytoskeleton. This process requires transmembrane signal transduction. The genes required for entry are carried on a 220 kb plasmid, one region of which encodes several polypeptides termed invasion plasmid antigens.[328] These Ipa proteins are required for full invasiveness and are immunogenic.[169] Some of the antigens are necessary for adhesion before the entry step. EIEC *E. coli* can multiply intracellularly within a very short period after entry. An important factor is the ability of EIEC to lyse its phagocytic vacuole soon after entry, unlike other invasive pathogens such as *Salmonella* and *Yersinia*.[327] The organisms multiply freely in the cytoplasm of the epithelial cell. Rapid, random movements occur that allow cell-to-cell spread without entry into the extracellular medium. This property requires the 120 kD product of the *virG* gene located on the virulence plasmid.[227]

43.5.4 Vero Cytotoxin-Producing *E. coli*

There is a family of Vero cytotoxins, VT, also known as Shiga toxins (Stx) or Shiga-like toxins because they share many properties with Shiga toxin, produced by *Shigella dysenteriae* 1.[167,275,339] Two major types of VT (Stx), VT1 (Stx1) and VT2 (Stx2), have been described based on toxin neutralization and DNA hybridization tests.[342] VT1 is neutralized by antibodies to Shiga toxin but VT2 toxins are not. Variants of VT1 and VT2 have been reported, with most variation seen in the VT2 toxins. Strains of *E. coli* O157 often produce a variant—designated VT2c—together with VT2.[334,387] The VT2 variants are neutralized to some degree by antisera against members of the VT2 family, although titers are usually highest with the homologous toxin.[145,182] Several combinations of VT toxins have been observed in strains of different serogroups, and some VTEC of animal origin also produce enterotoxins (ST or LT).[361] Details of the different members of the Vero cytotoxin family have been summarized.[339]

Vero cytotoxins are LT proteins comprising one A subunit and five B subunits. Like Shiga toxin, VTs cause an irreversible inhibition of protein synthesis in eukaryotic cells. There is cleavage of the N-glycosidic bond at position 4324 in the 28S ribosomal RNA of the 60S ribosomal subunit. A single adenine residue is released and aminoacyl-tRNA no longer binds to ribosomes.[194] The A subunit of VT is converted into a large N-terminal A1 fragment that has the enzymatic activity of VT and a smaller C-terminal A2 fragment. The B subunits bind the toxin to the receptors present on cells in certain tissues. VT1, VT2, and Shiga toxin bind to the glycolipid Gb3 which has a Gal α1-4 Gal β1-4 Glc ceramide structure.[237] With the VT2 variants VT2c and VT2e, there is preferential binding to Gb4 [Gal NAc β-(1$\rightarrow$3) Gal α-(1$\rightarrow$4) Gal β-(1$\rightarrow$4) Glc ceramide].[326]

The distribution of receptors in different tissues, such as the kidney, probably accounts for the specificity of toxin targets.[307] The presence of Gb3 on different cell lines also correlates with the susceptibility of these cells to VTs. Erythrocytes also contain Gb3, and it has been suggested that the relative binding of VT may affect the development of HUS in certain individuals.[378] This has been supported by the observation that HUS patients had lower Gb3 levels on their erythrocytes than patients infected with VTEC who did not develop HUS.[272] The sensitivity to VT of human glomerular microvascular endothelial cells is related to their high Gb3 level.[279] VT is cytotoxic for these cells and several other primary cell lines.[277,278,383] The level of receptor expression by glomerular endothelial cells is increased by cytokines.[398] Production of cytokines by macrophages and monocytes can be induced by the action of VT.[382]

Animal models have been tested with VTEC or purified VT,[168] but it appears there is no satisfactory model for the human disease. Experiments with rabbits showed that VT was an enterotoxin as well as a cytotoxin because it caused fluid accumulation in ileal loops.[276] Intravenous injection of VT1 and VT2 in rabbits resulted in diarrhea, anorexia, and neurological symptoms but no changes were seen in the kidneys.[307] Use of radioactive labeling and immunofluorescence demonstrated binding of toxin to tissues and these sites had glycolipid receptors. The mouse model has shown that there are significant differences in the lethal doses for different types of VT. Pigs also have been investigated as a model by several workers, and neurological effects as well as brain and kidney lesions were observed after intravenous injection but there was no damage in the intestine.[144] Recently, a disease in dogs termed cutaneous and renal glomerular vasculopathy (CRGV) has been shown to have characteristically similar lesions to those seen in children with HUS.[179] Further work is needed to define the etiology of CRGV and to determine whether the greyhound is an appropriate animal model for VTEC infection.

The production of VT usually is encoded by bacteriophages in strains of *E. coli* O157, and VT phages have been detected in some strains of other serogroups;[308,344,365] however, the genes for VT2e production are chromosomal in strains of porcine origin. Therefore, cloning of the toxin genes was performed with phage DNA for VT1 and VT2,[273,419] but with genomic DNA for VT2e.[408] Sequence data have shown the relationship between the members of the family of VT toxins. The VT1 sequence shows only three nucleotide differences from the Shiga toxin gene, whereas VT1 and VT2 have 58% nucleotide homology. Within the VT2 and VT2 variant genes, there is at least 70% homology but there are significant differences between the A and B subunit genes.[339,363]

VTEC possess properties other than VT production that may be important in their pathogenesis. Like the classical enteropathogenic *E. coli*, some VTEC have the ability to attach to and efface the microvilli of the intestine. Strains belonging to serogroups such as O5, O26, 0111, and O157 possess this property.[173,284,393,395] In a recent review, Buchanan and Doyle[46] stated that "*E. coli* O157:H7 did not form attaching and effacing lesions . . ." when referring to work with calves more than 3 weeks old that had been infected experimentally. However, work by Dean-Nystrom et al.[96] clearly demonstrated AE lesions in neonatal calves. It was concluded from this latter study that O157 VTEC are pathogenic for neonatal calves, and the effects are age-related. The genes required for the formation of AE lesions are located on a "pathogenicity island" or LEE region (locus of enterocyte effacement) on the chromosome in O157 VTEC and classical EPEC strains.[247] However, there are differences between the *eae* genes of an O157 VTEC and an EPEC strain.[19,427] The two genes share 86% overall nucleotide homology but within the 800 base pairs at the C-terminal end, the homology is only 59%. The sequence differences in the *eae* gene have led to the development of probes that have been used to examine strains of *E. coli* O157 and VTEC of other serogroups.[417] The product of the *eae* gene, termed intimin, may have different binding specificities depending on whether it is produced by an EPEC or a VTEC strain. Experiments in piglets showed that an O157 VTEC strain with a mutant *eae* gene did not attach closely to colonic epithelial cells.[102]

VTEC belonging to certain serogroups lack the *eae* gene;[415,416] however, some of these *eae*-negative strains may possess adhesive properties.[92,341,416] Studies with strains of porcine origin showed that many *eae*-negative VTEC produced fimbriae, such as F107, that appear to be important in their pathogenesis.[195]

Another putative virulence factor of VTEC is enterohaemolysin (E-Hly) production.[27,28] E-Hly is produced by virtually all O157 VTEC and by strains belonging to several other serogroups.[25,416] The plasmid-located E-Hly gene in the *E. coli* O157 strain 933 demonstrated approximately 60% homology with the *hly*A genes and *hly*C genes within the α-hemolysin operon.[332,333] The function of E-Hly is unknown but α-hemolysin causes release of interleukin1ß; one effect of this cytokine is to stimulate the production of the glycolipid Gb3. Cells containing Gb3 are thus more sensitive to the action of Vero cytotoxins.

The properties of EHEC include the production of VT, the ability to form AE lesions, and production of enterohemolysin encoded by the large plasmid.[228] Not all VTEC possess the LEE region required for AE lesions or the large plasmid but may produce other factors involved in their pathogenicity; therefore, at present, it is preferable to use the term VTEC (or STEC) rather than EHEC.

43.5.5 Enteroaggregative and Diffusely Adherent *E. coli*

Use of adhesion tests with tissue culture cells led to the definition of aggregative and diffuse adherence.[121] Animal studies with rabbits, rats, and piglets have shown histopathological lesions in the small intestine caused by EAggEC. The villi become edematous with necrosis of the tips, and there is infiltration of leucocytes and erythrocytes.[394,400] Eslava et al.[124] reported similar lesions in the ileum of infants examined on autopsy following an outbreak caused by EAggEC. The aggregative pattern of adhesion also is seen with cultured human colonic mucosa.[219] Fimbriae or fibrils on the surface of EAggEC have been demonstrated and may be responsible for the adhesion.[121,219,264] The genes encoding bundle-forming fimbriae AAF/1 have been cloned with plasmid DNA from strain 17-2, serotype 03:H2.[266] A second fimbrial antigen of EAggEC termed AAF/II has now been defined.[121,265] Plasmid-encoded, surface-located proteins are produced by EAggEC of several serogroups; those of serotypes O126:H27 and O44:H18 are antigenically similar and proteins of 18kDa have been identified.[72]

The diarrhea caused by EAggEC may be partially secretory. Several toxins have been described but their role in disease has not been elucidated. The ST enterotoxin, EAST1, has been detected in a rabbit *in vitro* intestinal model.[330,331] EAST1 is functionally similar to the STI toxins of ETEC in that it increases intracellular cyclic GMP. An LT toxin with a molecular weight of 120 kDa also has been described; this induced rises in intracellular calcium and calcium-dependent phosphorylation.[15] This protein reacted with antibodies to hemolysin, but further work is required to demonstrate any role for this protein in the pathogenesis of EAggEC.

The pathogenesis of the diffusely adherent *E. coli* is poorly defined. The adhesion among DAEC is heterogeneous, and the genes encoding diffuse adherence may be plasmid or chromosomally encoded.[22,32] Adhesion of DAEC to human ileal and colonic mucosa has also been reported.[426] The DAEC strain C1845 induced inflammatory lesions in the ileum of gnotobiotic pigs.[32] Mild necrosis, villous atrophy, and inflammation associated with an infiltration of neutrophils into the mucosa was observed; changes were most prominent in the large intestine. A number of toxins, including α-hemolysin and cytolethal distending toxin, have been described in DAEC strains but none is clearly established in pathogenesis.

43.6 PRINCIPLES OF DETECTION

Recent advances in DNA-based and immunological techniques have led to improved diagnostic methods for the detection of pathogenic *E. coli*.

43.6.1 Enteropathogenic *E. coli*

EPEC have been detected for many years using a range of antisera prepared with strains of the traditional enteropathogenic serogroups; however, this approach will not detect strains with properties of EPEC that do not belong to these serogroups. In addition, many strains within the EPEC serogroups do not possess the characteristics of EPEC and belong to other groups such as the ETEC or the EAggEC. "Classical" EPEC show localized adhesion to tissue culture cells. Strains are tested for adhesion in the presence of D-mannose; the cells are washed after 3 hours and incubated for another 3 hours before washing, fixing, and staining.[340,347] The 6-hour test is recommended because shorter times have led to problems in distinguishing patterns of attachment. The ability to form AE lesions can be tested using the fluorescence actin-staining assay.[217] Adhesion of EPEC results in the accumulation of actin under the attached bacteria, and this can be visualized using fluorescein-labeled phalloidin, which binds tightly to actin. Areas of fluorescence indicate the presence of AE *E. coli* such as classic EPEC.

Alternatives to these tissue-culture tests have been developed. An antiserum to an EPEC strain, absorbed with a plasmid-free derivative, has been used in an enzyme-linked immunosorbent assay (ELISA).[9] This assay detected strains giving localized adhesion, whereas nonadhesive strains and other types of *E. coli* were negative. DNA probe and polymerase chain reaction (PCR) tests have been developed from sequences on the EAF plasmid of strain E2348/69 (serotype O127:H6). The first probe was termed the EAF probe[14] and more recently a probe has been prepared from sequences encoding the bundle-forming pilus (*bfp*).[148] Classical EPEC strains hybridize with both the EAF and *bfp* probes. A DNA probe and PCR primers have been developed from the *eae* gene, one of the genes necessary for the AE lesion.[201] This *eae* probe detects classical EPEC but also many other strains, including some Vero cytotoxin-producing *E. coli*, including O157 VTEC.

43.6.2 Enterotoxigenic *E. coli*

The enterotoxins of *E. coli* were first demonstrated in animal models, particularly with the rabbit ligated-ileal loop technique. Tissue culture methods then were developed for detection of the LT enterotoxins. Culture filtrates of LT-positive strains produce morphological changes in Y1 mouse adrenal cells and Chinese hamster ovary (CHO) cells;[103,163] Y1 cells become rounded, and CHO cells elongate on exposure to LT. Vero cells also are sensitive to LT. These tissue culture assays should be confirmed by neutralization with specific antisera. Several immunological techniques are available for detection of LT based on production of polyclonal or monoclonal antibodies to LT.[166] ELISAs use antibodies to LT or CT, or alternatively GM1 ganglioside, the receptor for LTI. Other tests include the reversed passive latex agglutination test, a staphylococcal coagglutination assay, and the Biken test, which relies on precipitin detection with cultures grown on a special agar medium.[357] Sequencing of the LTI genes has led to the design of polynucleotide and oligonucleotide probes and PCR primers.[424]

For several years, the most widely used method for the detection of ST enterotoxin STI was the infant mouse test in which culture supernatants are in-

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jected into the stomach of the mice.[95] The ratio of gut weight to remaining body weight provides the measure of fluid accumulation. Immunological assays proved difficult to develop because of the nonimmunogenic nature of ST. Antisera to purified or synthetic toxin can be produced after coupling the toxin to a carrier. A competitive ELISA based on synthetic toxin and use of a monoclonal antibody now is available commercially.[346] DNA probes and PCRs using the STIa and STIb sequences also have provided convenient tests of detection for STI-positive ETEC.[424]

43.6.3 Enteroinvasive *E. coli*

EIEC usually belong to particular serogroups, and antisera have been produced for detection of such strains; however, most of these serogroups are antigenically related to serogroups of *Shigella* and therefore EIEC may be mistakenly identified as *Shigella*. The tests for the invasiveness of EIEC will also detect *Shigella*. The invasive ability of EIEC first was shown in the Sereny test in which bacterial cultures are placed in the eye of a guinea pig and the eye is examined later for keratoconjunctivitis.[351] A tissue culture method, developed as an alternative to the animal test, uses HEp-2 or HeLa cells.[340] After an infection period, monolayers are washed and reincubated in the presence of antibiotics and lysozyme to remove extracellular bacteria. After staining and fixing, cells are examined for the presence of intracellular bacteria. Invasive bacteria can also be identified in an acridine-orange staining method in which counterstaining with crystal violet quenches the fluorescence of extracellular but not intracellular bacteria.[253] An ELISA for detection of EIEC has been described that uses a polyclonal antiserum prepared against an invasive strain and absorbed with a noninvasive derivative.[285] Results of the ELISA correlated perfectly with the Sereny test.

The invasive ability of EIEC is determined in part by a large plasmid and sequences from this plasmid have been used for DNA probes and as PCR primers.[17,141]

43.6.4 Vero Cytotoxin-Producing *E. coli*

Evidence of VTEC infection can be provided by the isolation of VTEC including *E. coli* O157, by demonstration of specific VT in fecal or food samples, or by the presence in serum of antibodies to *E. coli* O157 lipopolysaccharide.[61,73,357] O157 VTEC can be detected by plating fecal specimens on MacConkey agar with 1% D-sorbitol instead of lactose as, unlike about 95% of *E. coli*, O157 VTEC do not ferment sorbitol rapidly.[130] Sorbitol nonfermenting colonies are tested for agglutination with an O157 antiserum or with an O157 latex agglutination kit. Modifications of this method have led to significant improvements in selectivity, in particular due to the incorporation of cefixime and tellurite into sorbitol-MacConkey agar (CT-SMAC).[428] The use of an enrichment culture step before plating has resulted in higher detection rates for O157 VTEC from fecal samples.[184] Detection of O157 VTEC in food samples usually requires an enrichment step. Two liquid enrichment media have been widely employed; these are a modified trypticase soy broth containing novobiocin and buffered peptone water with added vancomycin, cefixime, and cefsulodin.[61] Immunomagnetic separation (IMS) for *E. coli* O157 also provides increased sensitivity of detection.[39,61,71,205] Incubation of the food sample in the enrichment broth for 6 h at 42° C followed by IMS and plating on a selective medium such as CT-SMAC (sorbitol-MacConkey) provides a satisfactory method that can detect O157 VTEC at a level of less than 1 to 7 organisms per gram of beefburger.[39]

For the enumeration of *E. coli* O157 in foods, direct plating on SMAC or derivatives of this medium is unsatisfactory because the numbers present usually are too low to detect. Most probable number analyses have been used for enumeration.[421] Direct surface swab tests with the 3M Petrifilm Test Kit–HEC without enrichment have been used to evaluate beef carcasses for the presence of *E. coli* O157.[48] Recovery of *E. coli* O157 was consistently higher with phenol red sorbitol agar containing methyl umbelliferyl glucuronide (MUG) or 1% pyruvic acid than with other media, including SMAC.[5] The incorporation of a resuscitation period on trypticase soy agar containing catalase gave improved recovery of heat-stressed organisms in a comparison with direct plating on SMAC containing MUG.[246]

Several commercially available kits have been developed for the detection of *E. coli* O157, with particular emphasis on the testing of foods. The EHEC-TEK kit, an enzyme immunoassay, has been compared with a noncommercial method.[63] The sensitivity of the EHEC-TEK kit can be one organism in 25 grams but the assay was improved by use of buffered peptone water supplemented with vancomycin, cefixime, and cefsulodin in place of modified *E. coli* broth containing novobiocin. The kit was simple to use, but did not appear to be as sensitive as IMS followed by culture on CT-SMAC agar. Several commercial test kits for the detection of *E. coli* O157 strains in minced beef have been evaluated.[181] The tests were the Ampcor *E. coli* O157:H7 kit, 3M Petrifilm test kit-HEC, Dynabeads anti-*E. coli* O157, EHEC-TEK, and the Tecra *E. coli* O157 visual immunoassay. A sensitive and cost-effective method was concluded to be enrichment in modified *E. coli* broth containing novobiocin for 6 to 8 hours, followed by IMS and plating on CT-SMAC.

VTEC belonging to serogroups other than O157 can be detected by testing for the production of VT or the presence of VT genes.[356,357] The standard method for VT detection is a cytotoxic effect on a monolayer of Vero cells. Cytotoxicity due to VT should be confirmed by neutralization using antisera against VT1 or VT2 or with both antisera. Recently, commercial tests for VT detection have become available and these include ELISAs (Vero Test Kit, Microcarb and the Premier EHEC Kit, Meridian) and a reversed passive latex agglutination test (Unipath). One of the enzyme immunoassays detected VTEC in ground beef and milk at levels of one organism per gram of beef or per milliliter of milk.[3] The method required overnight enrichment before the immunoassay. The presence of VT genes can be detected by DNA probes in hybridization experiments or by amplification in PCRs.[204,294,420] Oligonucleotide probes and PCR primers have been developed from each of the VT2 variant sequences reported to date.[363,424] A commercial PCR test for the detection of VT-producing *E. coli* O157 has been developed by Qualicon. Enriched food samples are examined for the presence of sequences unique to *E. coli* O157:H7. The method requires preparation of DNA and has a reported sensitivity of 10^5 colony forming units per ml. Full evaluations are required for the commercial tests for VT detection and also the PCR–based systems.

43.6.5 Enteroaggregative and Diffusely Adherent *E. coli*

Adhesion to HEp-2 remains the standard method for the identification of strains belonging to these two groups. Enteroaggregative *E. coli* show a stacked brick pattern of attachment, whereas DAEC adhere randomly over the cell surface. As for testing of EPEC, a 6-hour period for the adhesion is recommended. Isolates producing hemolysin are difficult to assay for adhesion as they may destroy the monolayer. Alternatives to tissue culture assays are being developed and used for testing *E. coli* isolated from fecal specimens. The EAggEC probe pCVD432 was prepared from the plasmid in strain 17-2.[18] This probe identifies a plasmid that encodes EASTI production and the adhesive property. The pCVD432 probe was not developed from the genes encoding AAF/I but has been highly specific for detection of some EAggEC.[121] Two probes have been reported for DAEC. The plasmid pSLM852 carries a fragment from the *daa*C gene of strain F1845;[32] this probe is specific but has shown low sensitivity indicating that there are several adhesins for the diffuse phenotype. The second DA probe, termed AIDA-1, detects sequences that appear to be rare among DAEC.[22,200]

43.7 ROLE OF FOOD, WATER, AND THE ENVIRONMENT AS SOURCES OF DIARRHEAGENIC *E. COLI*

43.7.1 Incidence of EPEC, EIEC, and ETEC

Data on the incidence of EPEC, EIEC, and ETEC in foods and the environment are very incomplete. Investigations usually have been linked to outbreaks in the developed world, although some household-based studies on ETEC have been carried out in endemic regions. It is rare for a suspected food vehicle to be available for microbiological examination, so many associations between sources and disease have been based on epidemiological data.

Symptomatic and asymptomatic human carriers are probably the most important source of EPEC, EIEC, and ETEC that cause disease in man. In regions of high incidence, there appears to be acquisition of immunity with age so that a pool of pathogenic *E. coli* is available by fecal shedding to infect the young. Contamination of food occurs from food handlers or from contact with water containing human sewage. Infection of adult healthy volunteers with these groups of organisms requires a dose of at least 10^8 organisms, which would be consistent with the amount ingestible through contaminated food or water. However, in developing countries factors such as lowered gastric acidity and malnutrition may result in infection with significantly lower doses. Strains of ETEC do not produce significant quantities of enterotoxins when present in foods under most conditions, and ingestion of preformed toxins is unlikely to be a health hazard.[107]

There have been few surveys on the incidence of EPEC or EIEC in foods. In the one microbiologically proven foodborne outbreak of EIEC infection, the levels of organism in the contaminated cheese were between 10^5 and 10^7 g^{-1}.[242] Foods in the US unlinked to outbreaks have been surveyed for ETEC. One study on cheeses failed to detect ETEC,[152] but they were detected in 8% of a range of foods including cheese, beefburgers, sausage, and seafood.[322] The majority of the strains belonged to serogroup O149, an ETEC group associated with pigs, although some human-pathogenic ETEC serogroups, such as O78, O15, and O25, also were found. ETEC also have been detected in cheese and yogurt in Germany.[140]

Studies on the incidence of ETEC in food and water in developing countries have been relatively unsuccessful in identifying specific sources of infection. These investigations have included sampling of different water sources in the environment and in homes of diarrheal patients and food from recently prepared meals.[36] About 3% of samples of domestic water used for drinking, cooking, or washing utensils were found to contain ETEC in the homes of patients with diarrhea in Bangladesh.[36] Studies in Thailand showed that only 3 of 588 food samples contained ETEC.[117] Levels of ETEC contamination in drinking water ($<10^2$ ml^{-1}) and food (10^2 to 10^3 ml^{-1}) were substantially lower than that estimated to cause diarrhea in volunteer studies. A later study in Bangkok identified ETEC in 3% of 510 market foods and from 13% of cases of diarrhea in children under 5 years of age.[298] However, the serogroups isolated from food and human sources were different. A study in Brazil identified ETEC as the second most commonly isolated group of diarrheagenic *E. coli* in a survey of raw ground beef.[60]

Food items usually considered to carry risk of travelers' diarrhea include untreated drinking water and foods exposed to such water and not cooked before consumption. It has been suggested that the lower incidence of ETEC-associated diarrhea in British troops in the Arabian Gulf compared with their US counterparts was due in part to differences in sources of food supply.[414] Virtually all food supplies for British troops were military rations shipped from Britain, whereas US troops were supplied from Egypt and Southern Arabia.

43.7.2 Incidence of VTEC

The contrast between the epidemiology of VTEC and other diarrheagenic *E. coli* has resulted in a much wider knowledge of their occurrence in the environment, animals, and food. Although asymptomatic carriage by humans has been observed, the principal reservoir for these organisms is the intestinal tract of cattle and sometimes other animals. Contamination of meat occurs when intestinal contents are spilled at slaughter, and milk may become contaminated with feces. The low infectious dose of O157 VTEC means that foods that are subject to cross-contamination from raw meat, untreated milk, or animal feces may in turn become vehicles of infection if not subsequently cooked.

Presence of VTEC in Cattle and Other Animals

Many serogroups of VTEC have been isolated from the feces of healthy cattle. VTEC have been isolated from between 10% and 17% of healthy cattle, and in most studies a proportion of the strains has belonged to serogroups associated with human disease.[258,413] Some strains, such as those of serogroups O5, O26, and O111, may be pathogenic for cattle causing severe bloody diarrhea.[173,353] These serogroups also have been associated with human disease.[416] Although O157 VTEC have been found in diarrheal calves, their role in disease was not established,[282,376] and it is generally accepted that healthy cattle may harbour VTEC, including O157 strains, with no ill effect.

Early studies to survey the carriage of O157 VTEC reported their presence in less than 1% of healthy adult cattle in Britain and Germany.[70,258] Similar results were reported in Washington state where O157 VTEC were found in 0.28% of dairy cattle and between 0.33% and 0.71% of beef animals.[174] In a study in the US of animals possibly linked to human disease, O157 VTEC were isolated from 2.8% of heifers and calves and 0.15% of adult animals.[410] Studies in England have demonstrated that animals within a herd may have several separate episodes of shedding of O157 VTEC during the year.[250] Changes in management practices, including diet, were associated with fecal shedding but there appeared to be no link with grazing potentially contaminated pasture. In contrast, a US study had tentatively identified fertilization with slurry and a short waiting period between application and grazing as risk factors for the presence of O157 VTEC in herds;[174] however, a recent study did not show a higher prevalence of O157 VTEC in herds in which heifers were grazed on pastures treated with manure.[175] In Canada, studies on persistence of O157 VTEC on dairy farms in Ontario showed that shedding appeared to be transient and *E. coli* O157 was not isolated from several farm environment sites.[296] A report of a study of farms in Wisconsin showed that 7.1% of the dairy herds were positive for O157 VTEC with isolations of the organisms from 10 of 560 calves.[129] In England, up to 3.6% of cattle tested at slaughter carried O157 VTEC.[67,68] Not only did carcasses of animals that carried O157 VTEC become contaminated with the organism, but it also was spread to carcasses of O157 VTEC-negative animals adjacent on the processing line.[68] Studies by Chapman and colleagues[65] have demonstrated that 15.6% of rectal swabs from 4,800 cattle contained O157 VTEC. Maule[2] has examined the survival of O157 VTEC using a laboratory-based model system. Survival was demonstrated in soil cores for 150 days and in cattle feces for 90 days, but was much reduced in cattle slurry (20 days) and river water (15 days).

Strains of O157 VTEC have been recovered in England from the feces of sheep passing through an abattoir; 2.2% of the 1,000 samples were positive.[65,66] Studies of O157 VTEC in a flock of sheep in the US showed that the incidence was transient and occurred during the summer.[224] Wild deer also have been shown to be colonized by O157 VTEC that may have been acquired from range cattle sharing the same habitat.[305] Farmed deer in Britain were reported to carry O157 VTEC.[62]

Outbreaks of O157 VTEC infection have been linked to farm visits[354] and sporadic infections have resulted from direct contact with farm animals.[289,391] The farm environment is likely to be contaminated with O157 VTEC from cattle but the organisms can also be harbored by sheep and goats, which increase the likelihood of spread. Fecal material on skin or fur may be transferred directly to humans who touch the animals or indirectly by dogs.[377,390] A survey in Ontario screened dairy cattle and families living on dairy farms for VTEC including O157.[76] All herds were positive for VTEC on the first visit but only 0.45% of animals from 5% of farms carried O157 VTEC. None of the humans tested was stated to have been ill, although 8% harbored VTEC including strains of serogroups O5, O91, O103, and O157. In the working environment, transient carriage of VTEC occurred in clinically normal cattle and humans.

Although pigs carry VTEC, the serogroups identified rarely have been found in cases of human infection. A range of VTEC, but not O157, was harbored by healthy domesticated animals including cats and dogs, thus providing a further means of environmental spread.[26] There is no evidence that poultry are a natural source of VTEC including *E. coli* O157;[65] however, chicks became colonized with O157 VTEC after laboratory inoculation; they shed the organism for up to 11 months in their feces, and the shells of their eggs became contaminated.[335]

VTEC in Meat

Strains of VTEC have been recovered from between 9% and 25% of beef and beef products including sausages, beefburgers, and ground beef;[302,358,418] however, the serogroups identified did not include O157 VTEC. In a study examining a wider range of retail food samples (meat, poultry, and seafood), 17% of the samples were positive for non-O157 VTEC.[325] In contrast, Doyle and Schoeni[109] reported that O157 VTEC were present in 1.5% to 3.7% of samples of retail beef, pork, lamb, and poultry in North America. Part of their study was carried out in an area with a high incidence of O157 VTEC infection, and the possibility of cross-contamination before sale could not be excluded. A large study summarized by Griffin and Tauxe[156] reported O157 VTEC in 0.12% of raw beef samples.

Recent studies in Britain detected O157 VTEC in 5.9% of retail lamb products; the highest incidence, 7.2%, was in lambburgers.[64] In the same study, O157 VTEC strains were detected in 2.1% of retail beef products. A survey of raw meats in the Netherlands, using PCR, showed the presence of VT genes in 16.1% of samples of minced mixed beef and pork and minced beef samples.[180] None of the VTEC isolated from the PCR-positive samples belonged to serogroup O157. In the same report, only 2 of 770 meat samples were positive for O157 VTEC after testing by selective enrichment in mTSB with acriflavin, followed by plating on SMAC agar. VTEC but not strains of O157 were recovered from 20 of 105 samples of raw ground beef in Rio de Janeiro.[60] In this study, VTEC were the most commonly isolated group of diarrheagenic *E. coli*. A survey of red meat, poultry, and pork products in Argentina in 1996 isolated O157 VTEC from 0.4% of beef samples but not from beefburgers.[423]

Several isolations of O157 and other VTEC from foods have been the result of investigation of outbreaks or sporadic cases of disease. O157 VTEC were isolated in the US from hamburger patties in the first documented outbreak of HC[309] and from a batch of ground beef used to prepare burgers associated with a much larger outbreak.[20,157] In the latter investigation and in a report of O157 VTEC isolation in Britain,[421] attempts were made to quantify the number of organisms present in the meat samples. Most probable number analyses indicated a level of between 1 and 15 organisms per gram of uncooked beef. Keene et al.[213] reported an outbreak of O157 VTEC infection in the US that was traced to jerky, which was made from the drying of deer meat.

VTEC in Milk and Dairy Products

The importance of unpasteurized milk as a vehicle for infection was recognized in outbreaks in North America,[210,270] England,[69] and the Czech Republic.[31] A large survey failed to detect O157 VTEC in milk, dairy, and associated samples in the UK but the efficiency of the detection methods was recognized to be poor.[268] A more recent study in the US failed to detect O157 VTEC in soft and semi-soft cheeses, in the dairy environment, or in raw milk.[10] The largest milkborne outbreak to date was in 1994 in Scotland and involved heat-treated milk from a local dairy.[397] O157 VTEC indistinguishable from the human isolates were obtained from a pipe carrying milk from the pasteurization apparatus to the bottling machinery and from a discarded bottle machine rubber. Cheese prepared from raw cow's milk was the vehicle of an outbreak of O157 VTEC infection in Scotland[352] and in Northwest England in 1997.[80]

Strains of VTEC other than O157 have been associated with sporadic cases or outbreaks of infection. VTEC of serotype O22:H8 were identified in Germany in a patient with HUS and in milk from the patient's home and the supplying dairy.[38] An outbreak of gastroenteritis and bloody diarrhea in Montana was attributed to a VT2-producing strain of *E. coli* O104:H21.[56] There was a strong epidemiological link with consumption of one brand of pasteurized milk, but the implicated organism could not be cultured from samples obtained at the dairy or from rectal swabs taken from cattle on farms supplying the raw milk.

Incidence of VTEC in Other Foods

The range of food items implicated in outbreak and sporadic cases of VTEC infection has widened in recent years. In some instances, foods or beverages have been prepared from contaminated ingredients and organisms have not been inactivated in subsequent treatment. In 1994, dry cured salami was implicated as the source of O157 VTEC in an outbreak in the US.[388] At a similar time, mettwurst sausage contaminated with O111 VTEC caused an outbreak in Australia.[49,290,423] In these incidents, the survival of the VTEC strains during the fermentation and drying processes (see Section 43.8.2) provided a warning that the preparation of manufactured foods previously thought of as safe may have to be reconsidered. Identification of O111 VTEC in foods and in patients' stools posed particular difficulty in the Australian outbreak: unlike the use of sorbitol MacConkey agar for O157 VTEC, there are no simple tests for the rapid discrimination of O111 VTEC. An outbreak of O157 VTEC infection in the US was linked to consumption of apple cider, the unfermented juice of windfall apples.[23] This drink, because of its acidity, was not considered particularly hazardous and was drunk unpasteurized. It appeared that the apples were contaminated with cow manure in the orchard. Other outbreaks of O157 VTEC infection caused by drinking unpasteurized commercial apple juice occurred in the US and Canada in late 1996[57] and in Connecticut in October 1996.[58] In one of the outbreaks, O157 VTEC was isolated from an unopened container of the apple juice.[57] In June and July 1997 outbreaks of O157 VTEC infection occurred in Michigan and Virginia. Both instances were associated with eating alfalfa sprouts grown from the same seed lot.[59] In the very large outbreak in Sakai City, Japan, in 1996,[252,267] white radish sprouts were considered the most likely vehicle but this was not demonstrated microbiologically. In 1997 *E. coli* O157 was detected in white radish sprouts associated with sporadic infections.[423]

Many of the food vehicles implicated as sources of VTEC infection may have become cross-contaminated during preparation in the home or in catering establishments. Raw meat, meat products, or juices may contaminate other foods directly or via surfaces, utensils, knives, and hands to provide the low dose that may lead to infection. Foods possibly contaminated in these ways include salads, vegetables, fruit, mayonnaise, cooked meats, and sandwich fillings. Several institutional and community outbreaks have been linked epidemiologically to the last two items. Even when there has been no known contact with meat or milk, cases of O157 VTEC infection have been noted. One incident involved a strict vegetarian who fell ill after consuming garden vegetables that had been fertilized with O157 VTEC-containing manure from the family cow.[74]

Association of VTEC with Water

There have been sporadic cases and outbreaks of infection with O157 VTEC linked to consumption of water or its use for recreational purposes. Although epidemiological investigations may have indicated water as the vehicle of infection, microbiological confirmation usually has been lacking. Reasons for this may include insensitive detection methods and the likelihood that by the time water was suspected as a vehicle, it was too late to obtain samples relevant to the time of infection. There appears to be very little information on non-O157 VTEC in relation to water, and methods to optimize the isolation of these organisms require further development.

Laboratory studies summarized in the next section have shown that O157 VTEC can survive in untreated water. Infection has probably resulted from consumption of water from streams[269] or dams[197] that have been contaminated by cattle. Accidental ingestion of untreated water appears to have caused a sporadic case of infection in an individual who fell into a lake from a houseboat[269] and an outbreak among bathers swimming in a lake in Oregon.[212] Relatively small quantities of water probably were swallowed in these incidents, providing further evidence of the low dose of O157 VTEC needed for infection.

A survey of risk factors associated with O157 VTEC infection in Scotland indicated that 12% of cases had access to a private supply of drinking water. This study also showed that failure in the supply of potable water was a risk factor in 12% of cases.[79] Well water was suspected as the vehicle of infection in a large outbreak in Japan,[222] and water from little-used reservoirs may have caused a small community outbreak of O157 VTEC infection in Scotland.[99] In these cases the origins of the O157 VTEC were not identified, although in rural areas contamination of water supplies with cattle feces is likely. Contamination of the mains' water supply was implicated in a large outbreak of O157 VTEC infection in Cabool, Missouri (Table 43–2). The town received its water from a nondisinfected groundwater source. Two large water mains fractured in very cold weather and backflow of sewage may have occurred. Contamination of the public water supply was concluded to be the source of an outbreak of O157 VTEC and also of campylobacter infection in Scotland.[202]

Paddling pools may be associated with O157 VTEC infection in young children. In one incident in Scotland, a domestic pool filled from a main's water was probably fecally contaminated with O157 VTEC by one of the children.[44] A second outbreak involved a municipal pool in London at which disinfection procedures were inadequate.[183]

Occurrence of VTEC in the Environment

From the above discussion, there appears to be circumstantial evidence that surface waters may become fecally contaminated by VTEC including O157 from animals. There have been few studies, however, to examine environmental samples prospectively. One study in the Philadelphia area examined water samples from 15 environmental sites, including streams and reservoirs.[249] O157 VTEC was detected only in one sample, from a reservoir 45 km from the city; the origin of the bacteria was thought to be deer.

43.8 FACTORS AFFECTING SURVIVAL AND GROWTH OF DIARRHEAGENIC *E. COLI* IN FOODS

43.8.1 Diarrheagenic *E. coli* Other Than VTEC

Most studies on the survival and growth of pathogenic *E. coli* associated with diarrhea were performed in the late 1970s and early 1980s. They did not examine specific groups of *E. coli* and generally used various mixtures comprising ETEC, EPEC, and EIEC strains so that detailed information on the effect of environmental parameters is not available for each group. Some strains have been re-examined more recently for comparative purposes as part of studies on survival of VTEC, principally O157 strains. No studies have been published on the aggregatively adherent and diffusely adherent groups of *E. coli*.

The effect of concentration of sodium chloride (NaCl) and sodium nitrite, pH, and temperature on growth of a 10-strain mixture of EPEC, ETEC, and EIEC was studied in brain-heart infusion broth.[146] Levels of NaCl of 8% (w/v) or higher inhibited growth irrespective of other factors. In 6% NaCl, growth occurred between pH 6.8 and 5.6 at temperatures in the range of 15° C to 35° C but not at 10° C. At pH 6.8 or 6.2, the strain mix grew in any combination of sodium nitrite (0 to 400 mg ml^{-1}) and NaCl (0% to 4%) at temperatures between 15° C and 35° C. Overall, the *E. coli* mixture showed better survival than a cocktail of *Salmonella* strains, although it was impossible to determine whether the different *E. coli* pathogens were behaving similarly.

A recent study on O157 VTEC used strains of EPEC and EIEC for comparison in tests of acid tolerance.[11] When 10^4 cfu ml^{-1} of an EPEC strain of serotype O127:H6 were inoculated into artificial gastric fluid pH 1.5, there was 26% survival after 1 hour, a similar result to that obtained with some O157 VTEC (see Section 43.8.2) (data recalculated from Arnold and Kaspar[11] as actual percentage of survivors). Other EPEC and EIEC were undetectable in 2 hours or less, and a nonpathogenic strain survived for only 30 minutes. In the presence of food, the pH of the stomach contents probably would exceed 1.5, increasing the likelihood that EPEC and EIEC strains would survive to enter the small intestine.

Outbreaks of infection with diarrheagenic *E. coli* in which cheese was implicated as the vehicle of infection resulted in several studies of survival of strains during fermentation of dairy products and cheese making. In a model system,[137] a mixed inoculum of ETEC and EIEC strains grew by 1 to 3 $\log_{10}$ units in skim milk at 21° C and 32° C in the presence of different levels of lactic starter culture. However, growth of the *E. coli* strains was inhibited completely after 6 to 9 h by the lactic acid bacteria. Strains of *E. coli* O124, O125, and O128 grew during preparation of Camembert cheese from pasteurized milk inoculated with 10^2 cells ml^{-1},[288] but then declined as the pH of the products fell to 5.0 or below. After storage at 10° C, viable cells were undetectable after periods up to 9 weeks. Survival of pathogenic *E. coli* was better in cheese made by a modified process that resulted in a product with better buffering capacity that did not reach such a low pH.[297] Strains of *E. coli* could grow within the mycelial mat of *Penicillium camembertii* during cheese ripening at 10° C, indicating that cross-contamination of cheese surfaces could result in a product potentially hazardous to the consumer.[138] Studies on the preparation of hard cheese from artificially contaminated milk showed that strains of ETEC or EIEC multiplied by 2 to 3 $\log_{10}$ units during the manufacturing process, then declined gradually during ripening and storage but were still detectable after 4 to 7 weeks.[139,221]

43.8.2 VTEC

It is clear from the previous sections that VTEC, including strains of serogroup O157, may be present on raw foods. These include products of animal origin such as meat and milk and a range of other foods that may become cross-contaminated with VTEC. The low infectious dose of O157 VTEC implies that growth of the organism in food may not be a prerequisite for disease. The majority of data on survival and growth of VTEC relate specifically to *E. coli* O157:H7. The effects of temperature, pH, NaCl concentration, and carbon dioxide on growth, survival, and thermal inactivation have been studied, and a predictive model is available.[135]

In laboratory media such as trypticase soy broth, O157 VTEC multiplied rapidly between 30° C and 42° C with an optimum of 37° C.[108] Growth was poor at 44° C to 45° C and did not occur within 48 hours at 10° C or 45.5° C. The maximum and minimum temperatures for growth in brain heart infusion broth were obtained for 23 O157 VTEC isolates from different sources and 3 VTEC of other serogroups.[286] All strains grew at 10° C and a few at 8° C although the time to achieve a 1,000-fold increase in viable count ranged from 6 to 46 days. Growth has been observed between 6.5° C and 7.2° C in laboratory media when the conditions are favorable;[4] O157 VTEC may survive, in the absence of growth, at such temperatures in laboratory media (eg, trypticase soy broth) for 15 days at 5° C.[176] There appears to be some strain-to-strain variation in the maximum growth temperature but incubation temperatures in the 44° C to 45.5° C range, such as may be used for the detection of fecal coliforms in food, are unsatisfactory for the recovery of O157 VTEC.[106] Some VTEC, such as *E. coli* O26, may also be inhibited by high temperatures.[286]

Strains of O157 VTEC survive well in foods during freezing and frozen storage. In beef patties frozen at –80° C and stored at –20° C, there was little change in numbers of O157 VTEC after 9 months,[108] and 50% survival was reported in ground chicken meat stored at—20° C for 18 months.[82] Raw milk inoculated with O157 VTEC (10^1 to 10^3 cfu ml^{-1}) and frozen at –20° C yielded the organism after 63 days of storage.[10] The long-term survival of pathogens in frozen foods means that tracing the sources of contaminated raw materials may be virtually impossible. The conditions of freezing and thawing affect the degree to which microorganisms become injured and die, which may explain the relatively poor viability of O157 VTEC in one study in which only 9 of 23 previously positive samples were viable after 1 year's frozen storage.[255] Additives such as NaCl and sodium lactate decreased survival of O157 VTEC during freezing but did not result in their elimination after 18 months.[82]

Growth of O157 VTEC in foods at chilled temperatures has been reported. These included lettuce and cucumber at 12° C[1] and apple cider at 8° C.[430] Although numbers declined, O157 VTEC survived on lettuce at 4° C for up to 14 days,[1] and packaging of salad vegetables under a modified atmosphere (3% oxygen, 97% nitrogen) had little or no effect on the survival or growth of O157 VTEC at 4° C, 12° C, or 21° C. Under conditions that do not permit growth, O157 VTEC may survive refrigerated conditions for a time period long enough to be hazardous to the consumer. Strains of O157 VTEC inoculated at 10^3

cfu ml^{-1} did not grow at 5° C in milk or ground beef but remained viable.[287] There was growth at 8° C in milk or beef in which the background flora was low ($<10^3$ cfu ml^{-1}), but high background (10^5 cfu ml^{-1}) inhibited growth although the O157 VTEC survived. Consistent with these observations, growth of O157 VTEC at 8° C or 15° C was slower in raw milk than in pasteurized milk.[403] Pasteurized milk supported higher levels of O157 VTEC and viability was retained for longer than in raw milk. Milk that is contaminated after pasteurization and subjected to mild temperature abuse may contain O157 VTEC in sufficient numbers to cause infection. At 8° C, spoilage of pasteurized milk in the above tests was not detected after 28 days even though O157 VTEC was present at approximately 10^6 cfu ml^{-1}.

Several studies have demonstrated that O157 VTEC have no unusual resistance to heat. Doyle and Schoeni[108] reported *D* values at 62.8° C of 24 seconds and 42 seconds, respectively, for O157 VTEC and a strain of salmonella in ground beef. In pork and chicken homogenate, the *D* value at 64° C was 0.46 minutes.[24] The heat resistance of O157 VTEC was greater in fatty than lean ground beef.[236] The effect of product composition on the thermal death of O157 VTEC was demonstrated in ground beef, pork sausage, chicken, and turkey containing fat levels up to 30%.[6] The *D* values in the lowest fat products were lower than those in traditional beef and pork items. In milk, O157 VTEC strains are eliminated by pasteurization. In a study of thermal inactivation of pathogens in milk, exposure to 72° C for 16.2 seconds killed more than 10^4 O157 VTEC ml^{-1}.[89] At subpasteurization temperatures, O157 VTEC were more resistant to heat than *Yersinia enterocolitica* or *Campylobacter* species. Although O157 VTEC grew during the manufacture of cottage cheese, the bacteria were killed when the curd was cooked at 57° C for 90 minutes.[12]

Conditions for growth and growth phase affect heat resistance of O157 VTEC. Strains of VTEC were more resistant to heat when in the stationary phase.[389] Anaerobic growth resulted in *D* values at 55° C that were higher than for aerobically grown organisms.[262] The packaging of raw meat and vegetables under modified atmospheres could result in increased heat resistance of contaminating O157 VTEC. Brief exposure of O157 VTEC to elevated temperatures (42° C for 5 minutes) increased the *D* value at 55° C by a factor of 1.5 to 2.1.[262] It was speculated that mild heat shock that might occur during mishandling of food could compromise the ability of subsequent heat treatment to render the product safe. O157 VTEC may be more resistant to heat in sugar solutions rather than in salt at the same water activity.[135]

There are few data on the effect of water activity on the growth of VTEC. O157 VTEC grew in trypticase soy broth with salt concentrations up to 6.5% w/v corresponding to a water activity of 0.97.[151] Growth was inhibited at NaCl concentrations in excess of 8.5% w/v. Growth medium and temperature affect salt tolerance of O157 VTEC. Thus at 37° C, growth of O157 VTEC was inhibited at 8% NaCl but at 10° C there was inhibition at 4% to 6% NaCl.[81] It was concluded that the salt tolerance of O157 VTEC was similar to that of other *E. coli* serotypes and might be greater than that of *Salmonella* and *Campylobacter.*

Several outbreak investigations indicated that O157 VTEC infections had been associated with consumption of acidic foods that generally had been regarded as a low risk. Some of these were described in Section 43.4 and included live yogurt,[260] apple cider,[23,57] and mayonnaise.[211] These findings led to detailed study of the acid tolerance of O157 and some other VTEC in laboratory media and food. In Luria broth acidified with HCl to pH 2.5 or 3.0, there was no loss in viability of one O157 VTEC strain incubated at 37° C for 5 hours.[21] Five other O157 VTEC and several VTEC strains of serogroups O15, O22, O26, O111, and O165 were also acid tolerant but to a lesser extent. After 2 hours at 37° C, the percentage of bacteria remaining viable at pH 3.0 ranged from 26% to 80% and at pH 2.5 from less than 1 to 72%. In artificial gastric fluid at pH 1.5, most O157 VTEC survived for at least 1 hour.[11] For four strains, between 14% and 26% of the initial inoculum (10^4 cfu ml^{-1}) remained viable at the end of this period (recalculated from Arnold and Kaspar[11] as actual percent of survivors) and they were still detectable after 3 hours. A nonpathogenic *E. coli* control strain could not be recovered after 30 minutes. These results are consistent with the view that O157 VTEC and possibly other VTEC may not be killed rapidly in the stomach, contributing to the low dose required to cause infection. The minimum pH for growth is affected by the type of acidulant present; acetic acid is a more successful inhibitor than lactic acid and both were more effective than HCl.[90,151]

A strain of O157 VTEC was recovered from artificially inoculated apple cider at pH 3.6 to 4.0 held at 8° C for up to 31 days; however, at 25° C, the organism survived for only 2 to 3 days.[430] The expected shelflife of refrigerated apple cider was considered to be between 2 and 3 weeks so the pathogen could survive long enough to infect a consumer. The survival of the O157 VTEC in apple cider in these investigations was significantly reduced in the presence of 0.1% sodium benzoate, either alone or in combination with 0.1% potassium sorbate. In contrast, the addition of these preservatives was found to have little effect on survival in another study of O157 VTEC in apple cider.[254] The degree of acid tolerance was strain dependent. Survival of O157 VTEC has been demonstrated during preparation of yogurt from cow's milk containing 10^3 cfu ml^{-1}.[243] The pH values achieved during fermentation were in the range of 4.4 to 4.6. After 7 days at 4° C, there was a decrease in O157 VTEC level of less than 1 log_{10} unit cfu ml^{-1}. Similar results were obtained for traditional yogurt and a product that included *Bifidobacterium bifidum* in the starter culture.

Several reports from the US have examined survival of O157 VTEC in commercial mayonnaise and salad dressings. Federal regulations in the US, formulated to ensure destruction of salmonella at a time when unpasteurized eggs were used, required the product to contain 1.4% acetic acid at a pH of ≤ 4.1[429] or 0.7% acetic acid for reduced calorie mayonnaise. Studies have compared recovery of the organism from products stored at 5° C to 7° C or at room temperature (20° C to 25° C), considered to be the normal storage temperatures for these foods. At refrigerated temperatures, O157 VTEC survived for at least 35 days and up to 55 days in mayonnaise within the pH range 3.6 to 3.9.[407,429] At room temperature, O157 VTEC were undetectable between 72 hours and 96 hours after inoculation compared with a nonpathogenic strain of *E. coli* that survived for between 24 hours and 48 hours.[300,407] In one study however, the organism was recovered from mayonnaise for up to 21 days at 20° C, although a decline of up to a 1,000-fold was observed after 7 days.[429]

Abdul, Raouf et al.[1] showed that O157 VTEC could grow in salad mixtures containing beef and between 16% and 32% mayonnaise at pH 5.94 to 5.55 and were not inactivated in the product at 5° C. Although studies of O157 VTEC contamination of mayonnaise have often used unrealistically high inoculum levels, poor handling could result in cross-contamination by raw meat, meat juices, or unwashed utensils. Organisms might not be eliminated from mayonnaise, particularly if held at refrigerated temperatures. Dilution of mayonnaise during preparation of salads and sandwiches further diminishes the antimicrobial effect of the product.

Investigations on the survival of O157 VTEC in cooked or fermented meat products are relevant to outbreaks of infection with VTEC strains of serogroups O157 and O111 in which the vehicles of infection were salami-type sausages. There was growth of O157 VTEC inoculated into a slurry of cooked ground beef (pH 5.98) at 21° C.[1] Reduction of the pH with citric or lactic acid failed to stop growth but acetic acid did inhibit the organism. Isolates of O157 VTEC survived but did not grow in commercial sausage batter that was fermented to pH 4.8, dried, and stored for 2 months at 4° C;[151] the numbers declined from 10^4 g^{-1} to 10^2 g^{-1} by the end of this period. Similar results were obtained in the presence and in the absence of starter culture. At present no data are available on the survival of O111 VTEC in fermented meats. Although the rate of inactivation of O157 VTEC increases as a product becomes more acidic,[1] it is possible that the fermentation process as applied to items such as dry cured sausage may not be relied on to eliminate O157 and other VTEC and further controls may be necessary.

In the US, survival of O157 VTEC has been assessed on meat surfaces exposed to spray washing with water or solutions of organic acids. In one study on red meat slices, spraying with 0.5% to 1.5% acetic, lactic, or citric acid at 20° C or 55° C had no significant effect on the number of O157 VTEC still present on the meat surface after 13 days at 5° C.[42] Other reports indicated that washing with these organic acids could reduce the level of the organism by between 1 and 4 log_{10} units cm^{-2} but not eliminate it.[88,100,101] The effectiveness of treatment depends on the contamination levels, spray delivery method, temperature, tissue type, and acid concentration. A pilot-scale carcass washer that would mimic abattoir conditions was used to study reduction of O157 VTEC inoculated at 10^5 cfu cm^{-2} onto tissue surfaces from beef carcasses.[88] There was no significant difference in the effectiveness of acetic, citric, or lactic acids in reducing contamination. A concentration of 5% reduced the numbers by 1 to 2 log_{10} units, with removal from adipose tissue being better than from lean surfaces. Trisodium phosphate, which has been approved in the US for control of salmonella during poultry processing, also has been shown to reduce surface contamination of beef with O157 VTEC.[216] The above findings may have relevance for the control of VTEC contamination of carcasses (see Section 43.9.2).

Several factors affect acid tolerance of O157 VTEC. Survival of O157 VTEC in acid conditions was enhanced in late logarithmic and stationary phase and in cells that had been starved at 25° C.[11] Strains of O157 VTEC that were adapted to acid conditions by culture at pH 5 had increased resistance to lactic acid and survived better than nonadapted cells during sausage fermentation and in dry salami (pH 5.0) or apple cider (pH 3.4).[234] Several acid tolerance systems appear to contribute to the survival of O157 VTEC in the environments of the stomach (pH 1 to 3) and in the intestine (pH 4.5 to 7) in the presence of fatty acids.[235] Once induced, these systems persisted during storage of the organisms at 4° C for up to 28 days. Strains of O157 VTEC adapted to pH 5 were recovered in greater numbers than nonadapted cells after inoculation into ketchup (pH 3.6) and storage at 5° C.[392] There was strain-to-strain variation in the survival times but, generally, adapted cells were recovered for 5 days.

Two studies have examined the survival of O157 VTEC strains in meats exposed to gamma irradiation. Factors affecting inactivation of the organism included temperature, atmosphere, fat content, and radiation dose. An inoculum of $10^{4.8}$ cfu O157 VTEC g^{-1} of ground beef was reduced to less than 10 cfu g^{-1} by a radiation dose of 0.75 kGy, and the organism was undetectable after exposure to more than 1.5 kGy.[384] Clavero et al.[77] calculated from irradiation inactivation curves that a dose of 2.5 kGy would kill $10^{8.1}$ O157 VTEC g^{-1} of ground beef.

Survival of O157 VTEC strains in drinking water has been examined in response to epidemiological evidence of waterborne outbreaks.[306,375] Well water collected from the water source implicated in the outbreak in Cabool had a coliform count of less than 1 organism $(100\ ml)^{-1}$. Samples were inoculated with 10^6 to 10^7 cfu ml^{-1} of O157 VTEC strains from either the Cabool outbreak or from a beefburger. There was no significant reduction in viable counts of the strains for 7 days at 5° C or 20° C. After this time, death occurred more rapidly at 20° C with a 5 $\log_{10}$ reduction after 35 days; strains survived at 5° C for at least 70 days. There appeared to be no significant difference between survival of the O157 VTEC strains and a control *E. coli* strain isolated from river water. Strains of O157 VTEC are as susceptible to the chlorination treatment used for drinking water as are other *E. coli.*[131]

43.9 CONTROL AND PREVENTION OF INFECTION

Some of the factors that affect the control of infections caused by diarrheagenic *E. coli* have been mentioned in previous sections on the epidemiology of infection and the occurrence of the organism in food and the environment. Although many general preventive measures are applicable to all the groups of diarrheagenic *E. coli,* the zoonotic nature of VTEC infections presents specific problems.

43.9.1 Diarrheagenic *E. coli* Other Than VTEC

The principal source of EPEC, ETEC, and EIEC and possibly the newer groups of *E. coli* involved in human disease appears to be the intestinal tract of affected individuals and asymptomatic carriers. In areas where disease is endemic, control in the population is achieved by improvements in sanitation, particularly the provision of safe drinking water and the disposal of sewage to avoid contamination of water supplies. The practice of fertilizing crops with untreated human sewage should be avoided. Control of flies may reduce the likelihood that they may act as vectors of infection. In developed countries where good sanitation is the norm, it is important to ensure adequate standards in recreational facilities such as campgrounds and parks.

Hygienic practices in handling and preparation of foods are of great importance. Education of food workers should include proper personal hygiene, particularly hand washing, and safe techniques that ensure adequate cooking and storage of foods under conditions to avoid bacterial multiplication. These measures are particularly important in relation to recently weaned children. The practice of prolonged breast feeding in many developing areas provides important protection against disease and avoids the use of potentially contaminated water and feeding equipment. Unchlorinated water should not be used for cleaning utensils and food contact surfaces or for cleaning or cooling in commercial food production units. Control of travelers' diarrhea is achieved by avoidance of potentially contaminated foods that are eaten raw, including salads, unwashed fruit, and unheated milk. Travelers to countries where infections due to diarrheagenic *E. coli* are common should rely on hot food and drinks or on water in sealed bottles and avoid adding ice to beverages.

Vaccines have been proposed for the prevention of infection with some groups of diarrheagenic *E. coli* strains. In the 1970s oral vaccines based on EPEC serogroups including O55, O86, and O111 were tested in field trials in infants but the efficacy of the vaccines was insufficient for routine use.[256,301] Future developments are more likely to be based on factors involved in adhesion of EPEC, such as bundle-forming pili and intimin. Numerous vaccines have been developed against infection by ETEC including live preparations and nonliving antigen mixtures that may comprise toxoids, inactivated whole cells, and purified surface antigens. Two candidate vaccines have incorporated the B subunit of CT, and one of these also contained killed whole cells of *Vibrio cholerae.*[78,374] Problems in the successful development of vaccines against human ETEC have arisen from the diversity of serogroups and the number of different colonization antigens involved and also from economic factors. This contrasts with the widespread use of vaccines to control ETEC infections in piglets and calves.[198] Studies on EAggEC and DAEC have not yet revealed suitable antigens on which vaccines could be based.

Successful treatment of *E. coli*-associated diarrhea depends on early correction of fluid and electrolyte imbalance. In the developing world, use of oral rehydration therapy is of particular importance in infection caused by ETEC. In hospitals and nurseries, measures to avoid spread of infection from patient to patient include barrier-nursing techniques and isolation of cases. The widespread environmental contamination may require closure of wards for disinfection and screening of carers for carriage of the *E. coli* pathogen.

Widespread use of antimicrobial agents for treatment of disease due to EPEC and ETEC has been questioned. The practice will lead to spread of antibiotic resistance, which is increasingly common in both these groups of organisms. In addition, some of the antimicrobial agents employed have high toxicity, particularly to young children most at risk of infection. Prophylaxis with cotrimoxazole, trimethoprim, or ciprofloxacin may reduce the duration of travelers' diarrhea.[35,114,122]

43.9.2 VTEC

Measures for control and prevention of VTEC infection should take account of the clearly identified reservoir of the organism in the intestinal tract of cattle and other food or domesticated animals and the low dose required for infection, at least with O157 VTEC. It is necessary to address and break the three major transmission routes identified as foodborne infections, acquisition of disease through direct or indirect contact with animals, and person-to-person spread.

The control of VTEC in food in Britain has been addressed recently in detail.[4] This report stated that it must be accepted that VTEC may be present on or in raw foods, particularly meat and milk that may be contaminated by fecal slurry. The factors affecting fecal carriage and excretion of VTEC in cattle appear complex, and management practices appear to influence prevalence in herds. Further investigations are needed to assess methods to minimize carriage and excretion in beef and dairy animals. The Richmond Committee on red meat production[304] made recommendations on slaughtering that are highly relevant to control of VTEC. These related in particular to reducing contamination of carcasses with gastrointestinal contents and the use of equipment that combines high throughput with ease of cleaning. Microbiological monitoring of key equipment was seen as a check of cleaning and disinfection processes.

There have been several studies from the US on the use of carcass washes particularly with hot organic acids (see Section 43.8.2), but the relative resistance of O157 VTEC to low pH may limit their usefulness. The US Food Safety and Inspection Service has approved the use in beef processing plants of a steam vacuum sanitizing system delivering water at 88° C to 94° C onto carcass surfaces. In laboratory experiments O157 VTEC applied in fecal slurry on beef carcass samples were reduced from a level of 7.6 $\log_{10}$ cfu cm^{-2} to about 2.1 $\log_{10}$ cfu cm^{-2} by this treatment.[104] A further study[105] examined the fate of O157 VTEC on carcass samples that had been spray washed at 32° C and then stored at 5° C for 21 days. Washing with organic acids or trisodium phosphate reduced the initial inoculum from 4 $\log_{10}$ cfu cm^{-2} to less than 1.3 $\log_{10}$ cfu cm^{-2}, which was maintained over the storage period. In contrast, there was growth of O157 VTEC on water-washed beef tissue after 2 days' storage.

The application of the Hazard Analysis Critical Control Point (HACCP) system will provide a structured approach to the prevention or limitation of VTEC in the food supply. This approach is based on use of data on the occurrence, survival, and growth of VTEC in foods to identify stages in food production, manufacture, storage, or supply to the consumer that pose a hazard. One such critical point is the supply of raw materials. Control may be achieved by purchase from hygienic, inspected sources according to an established specification to maintain quality assurance. This is particularly important for the production of minced meat items such as sausages and beefburgers in which organisms contaminating meat surfaces become distributed throughout the final product. Hazards associated with these foods could be further reduced by informative labeling indicating safe storage, handling, and cooking procedures.

Following an investigation of the central Scotland outbreak, the Pennington group published a detailed final report.[291] There were 32 recommendations in eight major areas covering all aspects of food production and consumption from "farm to fork." The present, and also the last, UK government accepted all the recommendations.

A different approach to control of O157 VTEC in raw ground beef has been adopted in the US. The Food Safety and Inspection Service declared in 1994 that *E. coli* O157:H7 was an adulterant in this product and that contaminated lots be excluded from commerce and either destroyed or reprocessed to kill the organism.[196] In 1997, approximately 25 million pounds of ground beef produced at the Hudson Foods, Columbus, Nebraska, plant were recalled.[225] An outbreak in Colorado was associated with consumption of burgers from particular lots of frozen beef patties produced by Hudson Foods. Protocols for sampling at production plants, laboratory testing, and regulatory procedures have been established, and the Inspection Service has legal authority to impose these measures. In view of the vast size of the ground beef industry in the US and the difficulty of realistic sampling, the value of these procedures is uncertain unless controls also are imposed to minimize initial contamination at farms and abattoirs.

The continued availability of raw cow's milk in England, Wales, and Northern Ireland remains controversial. Raw milk has been associated with O157 VTEC infection as well as with outbreaks caused by *Campylobacter* and *Salmonella* and may pose a particular threat for vulnerable groups including children. A recent report[4] proposed reconsideration of the legislation that would require obligatory pasteurization of milk, as is practiced in Scotland. Studies on survival of O157 VTEC at low pH have indicated that products such as cheese and yogurt made from raw milk must also be subject to more stringent control.

Elimination of VTEC by heating is one of the major control points in the food chain. Pasteurization of milk by procedures already defined by legislation should ensure a product that poses no threat to human health. However, one recent incident has emphasized the need to prevent postpasteurization contamination. There have been extensive investigations on cooking procedures to destroy VTEC in minced meat products, particularly O157 VTEC in fresh and frozen beefburgers.[4] Most data suggest cooking so that all parts of the burger are maintained for 2 minutes at 70° C, or equivalent values, until juices run clear and there are no pink bits inside. However, factors such as meat composition, size, thickness, and whether the item is chilled or frozen will affect adequate cooking times. Raw beefburgers and other minced meat products should be supplied with cooking instructions adequate to destroy VTEC and that take account of different cooking techniques that may be used.

As described in previous sections, outbreaks of infection with O157 and other VTEC have been associated with fermented but unpasteurized foods such as apple cider and sausage. The use of minimally contaminated starting materials and microbiological monitoring are essential to reduce hazard because the fermentation processes involved may not be relied on to provide control. Preservatives such as sodium benzoate may increase the safety of apple cider.[430]

The potential for cross-contamination is a major concern throughout the chain of food production and particularly in the domestic kitchen and catering environments. Guidelines for avoiding direct and indirect cross-contamination are available from a number of sources.[4,291] As it is unlikely that VTEC will be eliminated from the food chain, education and training in food hygiene are important for commercial food handlers and consumers. Increased public awareness generated by government and retailers could reduce sporadic cases and small family outbreaks that may be the result of poor food handling in domestic kitchens.

Recent reports of VTEC carriage by a range of animals in addition to cattle have indicated that animal contact is a risk factor for human infection. Farmers have a responsibility to ensure a safe working environment and appropriate control measures. More studies are required to assess the prevalence of VTEC in adults working with livestock on farms and in abattoirs. The observance of simple hygiene procedures by members of the general public attending farm visitor centers is the most important preventive measure for infection with VTEC.[91] Handwashing facilities should be easily accessible and their use strongly encouraged before eating and drinking. Visitors should be made aware of potential hazards by leaflets or notices. Young children should be supervised to ensure adequate hand washing. Designated eating areas must be clearly separated from enclosures with animals and should, where possible, be outside the main farm areas.

Recommendations for the control of infections caused by VTEC in England and Wales are based on those for *Shigella sonnei* infections. They take account of the potential severity of disease, the low infectious dose, and the particular hazard to young children.[372] The importance of good hygiene practices is stressed, particularly the provision of toilet facilities, hand washing, and formal procedures for cleaning of nursery and school environments and equipment. It has become clear that O157 VTEC may be harbored asymptomatically or may be excreted for long periods after onset of symptoms. Although generally, individuals may return to school or work after diarrhea has ceased, some groups pose a special risk of spreading infection. These include food handlers, health care workers, preschool children attending a daycare facility, and older children or adults who are unable to implement adequate personal hygiene for personal or environmental reasons. Exclusion of the individuals from the first three groups has been recommended until negative cultures have been obtained from two consecutive fecal samples obtained 48 hours apart. Contacts of persons in the special groups should also be excluded until the household case is asymptomatic.

Infection with VTEC is usually self-limiting, and the value of antimicrobial therapy for disease caused by O157 VTEC has not been established. The duration of symptoms was not reduced by treatment and there may be an increased risk of HUS.[422] Evidence from North America, England, and Wales suggests that resistance to antimicrobial agents is becoming more prevalent in O157 VTEC strains;[215,386,412] however, multiple resistance, such as encountered in *Salmonella* and *Shigella,* is very rare. The use of antimotility agents to treat bloody diarrhea caused by O157 VTEC was significantly associated with progression to HUS.[75] There are no vaccines available for the prevention of VTEC infection.

43.10 CONCLUDING REMARKS

Diarrheagenic *E. coli* form a complex group of organisms that fall into different classes with contrasting properties. Different reservoirs, modes of transmission, and epidemiology pose various problems both clinically and with regard to the role of foodstuffs. It is important to recognize the continued threat posed by *E. coli* as a diarrheal pathogen. Whereas the importance of EPEC, ETEC, and EIEC has declined in developed countries, VTEC have emerged and may constitute a more far-reaching problem because of their presence in food animals. It appears likely that EAggEC will also be recognized as a significant cause of diarrheal disease although much more work is needed on these organisms.

There is a need for better tests to identify diarrheagenic *E. coli* in clinical specimens and foods. This is particularly relevant for VTEC strains other than O157. Lack of tests for these organisms has hindered assessment both of their prevalence and their status as pathogens. In the absence of convenient biochemical markers, tests for non-O157 VTEC and EAggEC may be based on properties associated with pathogenesis. At present it is likely that there is considerable under-recognition of sporadic cases and outbreaks of diarrhea due to *E. coli*, and this will continue as long as many tests are available only in reference laboratories.

In relation to the food chain, further studies are required to identify reservoirs and vehicles of infection with diarrheagenic *E. coli*. For EAggEC, the role of foods is not yet established and it is not known whether these organisms are harbored by agricultural livestock. Although these aspects have been studied in relation to O157 VTEC, it has become clear that spread of the organism can be much more complex than the originally identified link with consumption of ground beef and raw milk. Further studies should include non-O157 VTEC and consider factors affecting animal carriage, the application of hygiene practices in the early stages of red meat production, and the search for other vehicles of infection.

The relevance of diarrheagenic *E. coli* to the food industry centers at present on VTEC. All sectors should adopt a HACCP-based approach for control of these organisms. Manufacturers should be aware that modifications of processes, possibly in response to economic factors or the demands of modern consumers, may have microbiological significance with respect to the survival of contaminating organisms. Education and training of workers should have increased priority at all levels of food production. However, good communication with the consumer may be one of the most effective approaches for control of diarrheagenic *E. coli* and other enteric pathogens. Advice should be available on health and hygiene and clear guidelines produced on food preparation and cooking communicated by means of informative labelling. These measures could be put in place by the government, consumer organizations, and the food industry.

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44 *Listeria monocytogenes*

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44.1 INTRODUCTION

The history of *Listeria monocytogenes* dates back to at least 1918, when Dumont and Cotoni[125] isolated from cerebrospinal fluid (CSF) an organism that was deposited at the Pasteur Institute in Paris and later identified in 1940 as *L. monocytogenes*.[394] Murray et al.[367] described the organism first in 1926 and named it *Bacterium monocytogenes* because it caused a mononuclear leucocytosis in rabbits. Pirie,[403] a few years later, isolated a bacterium from a gerbil in South Africa, which he named *Listerella hepatolytica* in honor of Lister; therefore, the name listerellosis was used to describe the disease process caused by this organism for some time. When it was discovered that *Bacterium monocytogenes* and *Listerella hepatolytica* were the same organism, and that the name *Listerella* already had been given to a group of slime molds, it was decided to change the name of *Bacterium monocytogenes* to *Listeria monocytogenes*.[403] The emergence of this "awkward and unnoticed adolescent"[211] into an important foodborne pathogen has been quite dramatic. Since

1985, when the organism was identified as the causative agent in a large (142 cases with 48 deaths) foodborne outbreak involving Mexican-style soft cheese,[306] it has been a source of continual problems for the food industry and regulatory agencies. The estimated annual costs and productivity losses for human listeriosis in the United States (US) were recently given as $209 to $233 million.[521] These figures, however, do not include the economic losses to the various food companies caused by the recall of *L. monocytogenes*-contaminated product, as well as *Listeria* control costs. In one survey involving 17 meat producers, *Listeria* control costs totalled $9.3 million, or roughly 0.1% to 0.2% of sales.[427] Thus, the importance of this organism not only from a human health, but also from an economic standpoint, has led to a dramatic surge in interest among food manufacturers and regulatory bodies. The following sections will discuss some of the important features of this foodborne pathogen.

44.2 TAXONOMY AND PROPERTIES OF *L. MONOCYTOGENES*

44.2.1 Taxonomy

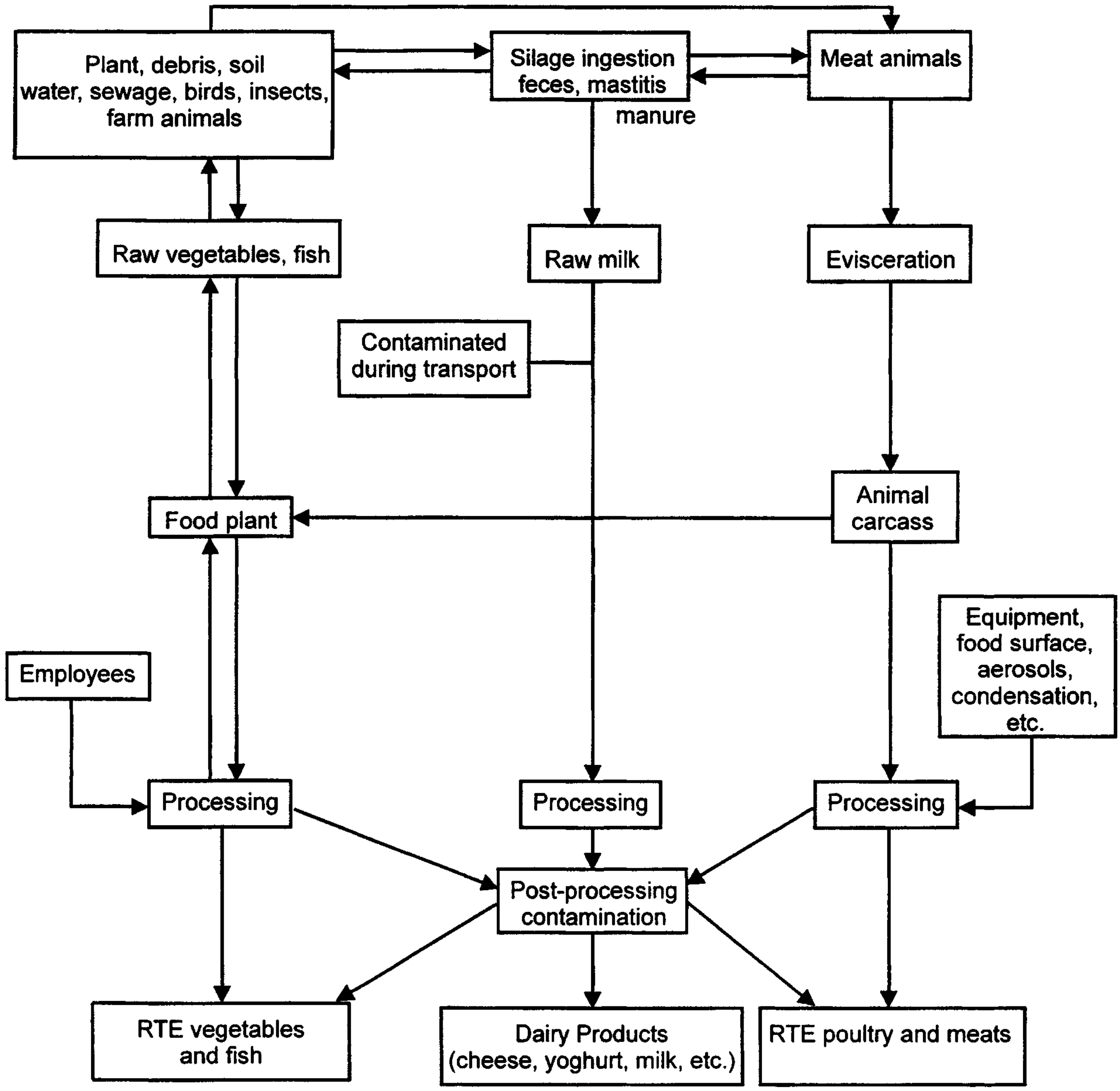
In the seventh edition of *Bergey's Manual of Determinative Bacteriology* (1957), *L. monocytogenes* was listed in the broad grouping of the family *Corynebacteriaceae*, mainly because of similarities in morphology. In the eighth edition,[452] the organism was placed in a grouping (with two other genera, *Caryophanon* and *Erysipelothrix*) called "genera of uncertain affiliation." At this time, three other species were included in the genus, namely, *L. denitrificans, L. murrayi,* and *L. grayi. L. denitrificans* also has now been moved to another genus and is referred to as *Jonesia denitrificans*,[431] while *L. murrayi* and *L. grayi* were merged to form a single species: *L. grayi.* The use of 16S ribosomal RNA partial sequencing showed that *L. monocytogenes* belonged within the *Clostridium* subbranch along with *Brochothrix*, *Staphylococcus*, *Streptococcus*, and *Lactobacillus.*[317] These findings were taken into account in *Bergey's Manual of Systematic Bacteriology* published in 1986,[453] where the genus *Listeria* was placed in a section called "regular, nonsporing, Gram-positive rods," with six other genera, including *Brochothrix*, *Lactobacillus*, *Kurthia*, *Renibacterium*, *Caryophanon*, and *Erysipelothrix.* In the latest (1994) edition of *Bergey's Manual*, *L. monocytogenes* was placed in Group 19, again referred to as "regular, nonsporing, Gram-positive rods."[234] The only genus added to this group in this edition was *Carnobacterium.* Group 19 contains genera placed together mainly for convenience in strain identification, and the genera share only a few similarities in terms of physiological or morphological characteristics.[234]

Evidence obtained from sequencing the flagellin, listeriolysin, and invasive-associated protein genes has provided evidence that *L. monocytogenes* exists in at least three evolutionary lines, with a strong degree of conservation within those lines.[419] Most isolates, however, belong to only two of the sequence types for which there is a correlation with flagellar antigens, as well as grouping based on pulsed-field gel electrophoresis (PFGE),[56] multilocus enzyme electrophoresis (MEE),[39] randomly amplified polymorphic DNA (RAPD) typing,[335] and restriction fragment length polymorphism (RFLP) analysis.[512,536] In most studies, division I contains serotypes 1/2a, 1/2c, 3a, and 3c, whereas division II includes serotypes 1/2b, 3b, 4b, 4d, and 4e. Serotype 4a isolates appear to form a distinct homogenous group,[419] while serotype 4b strains can be further divided into at least two groups.[131,536]

44.2.2 General Properties

Listeria species are short, Gram-positive, nonsporeforming, facultatively anaerobic rods, which are motile by means of a few peritrichous flagella. Motility occurs from 20° C to 25° C, but not at 37° C. Transcription of the gene encoding flagellin is pronounced at 22° C, but is undetectable at 37° C.[119] Interestingly, *L. innocua* produced larger amounts of flagellin at 37° C than *L. monocytogenes* and often is motile at this temperature.[270] This suggests that significant differences exist between the two species in their regulation of flagellin gene expression at 37° C.

Listeria spp. are psychrotrophic organisms, capable of growing at refrigeration temperatures; they are a concern in extended shelf-life, refrigerated foods. The ability of the organism to grow over a wide temperature range in acidic environments, as well as in the absence of or at very low amounts of O_2, enables it to multiply in many environments. The organism is widespread (Figure 44–1) and can be found in many locations such as water, silage, sewage, slaughterhouse waste, milk of normal and mastitic cows, and human and animal feces.[142,319,505] A seasonal variation in human fecal carriage of *L. monocytogenes* has been reported.[319] It has also been suggested that the plant-soil rhizosphere may be a natural habitat for *Listeria* spp.[505]


Plant, debris, soil water, sewage, birds, insects, farm animals
Silage ingestion feces, mastitis
manure
Meat animals
Raw vegetables, fish
Raw milk
Evisceration
Contaminated during transport
Food plant
Animal carcass
Equipment, food surface, aerosols, condensation, etc.
Employees
Processing
Processing
Processing
Post-processing contamination
RTE vegetables and fish
Dairy Products (cheese, yoghurt, milk, etc.)
RTE poultry and meats

RTE = ready-to-eat

Figure 44–1 Overview of Transmission of *L. monocytogenes*

44.3 CHARACTERISTICS OF THE DISEASE

44.3.1 Incidence and General Characteristics

Most cases of human listeriosis appear to be sporadic, although a portion of these sporadic cases may be common source clusters that were previously unrecognized.[80] The true rate of human listeriosis worldwide is underestimated because most available data are based on voluntary reporting. The reported incidence of listeriosis varies from 0.2 to 8.3 cases per million population, with most cases being reported in industrialized countries (Table 44–1).[428,430] Higher incidence rates, however, have been reported in some countries.[142,378] Although it is considered that as many as 80% to 90% of human listeriosis cases are linked to the ingestion of contaminated food, demonstration of foodborne transmission occurs infrequently, and generally is due to unusual circumstances.[63,306] Listeriosis can also be acquired

Table 44–1 Reported Human Listeriosis Cases[a] in Selected Countries

Year	*Canada*	*US (5 areas)*[b]	*UK*[c]	*France*[d]	*Australia*
1987	44				
1988	63				
1989	63	151			
1990	49	147	116	305	
1991	49	117	127	387	44
1992	32	88	106	737	38
1993	56	84	102	489	53
1994	46		112	336	34
1995	54		85	301	58
1996			116[e]		

[a] 80% to 90% of cases are thought to be food-related.

[b] Area of 19.1 million.

[c] Personal communication, Dr J. McLauchlin.

[d] Personal communication, Dr J. Rocourt.

[e] Provisional total.

by direct contact with infected aborteous material from animals,[437] by nosocomial cross-infection,[151,339] and by means of aerosolization. The majority of human cases occur in individuals who have an underlying condition that leads to suppression of their T-cell mediated immunity. Skogberg et al.[465] found that 43% of 74 cases of systemic listeriosis in Finland had received immunosuppressive therapy within 1 week before the onset of listeriosis, and concluded that prior immunosuppressive therapy is an important predisposing factor for listeriosis. Table 44–2 lists the most common underlying conditions of cases of listeriosis reported worldwide. Patients with acquired immunodeficency syndrome (AIDS) have been reported to be 280 times[448] more susceptible to listeriosis than is the general population, and *L. monocytogenes* is now recognized as an important opportunistic pathogen in human immunodeficiency virus (HIV) infected individuals.[264]

Using data collected from France and the US, high-risk individuals can be ranked by decreasing risk as follows:[429] organ transplant recipients (200 cases per 100,000 patients); patients with AIDS (115 cases per 100,000 patients); HIV-infected patients (52 cases per 100,000 patients); pregnant women (4.7 to 30 cases per 100,000 live births); cancer patients (13 cases per 100,000 patients); and the elderly (1.4 cases per 100,000 individuals over 65 years old). Several factors may increase the susceptibility of individuals to listeriosis, for example, the use of antacids and laxatives.[229,448] The decreased gastric acidity resulting from the use of antacids may increase the number of organisms that survive passage through the stomach, ultimately resulting in a lower minimum infectious dose. Laxatives are known to alter transit time and modify the intraluminal environment of the gastrointestinal (GI) tract.

It is not known why the incubation period of listeriosis varies so much (more than 24 hours to 91 days). It is possible that the dose of organism ingested, the host immune system, and any intercurrent viral or bacterial infection all play some role in determining the period of onset of the illness after initial exposure to the organism.

Worldwide figures for human listeriosis reported for the years 1989 and 1990 show that 43% and 34.3% of the total cases reported were maternal and neonatal infections, respectively.[428,430] During pregnancy, maternal immune reactions are suppressed. Studies with mice have shown an apparent decrease in *Listeria*-specific cellular reactions during pregnancy (ie, there were abnormalities in the spleen dendritic cells).[280] The proportion of cases involving pregnant women or various groups of immunosup-

Table 44–2 Underlying Conditions of Adults with Listeriosis

Main Predisposing Health Conditions (222 patients)	
Malignancy: (lymphoma, sarcoma, leukemia, etc.)	135 cases (61%)
Organ transplantation	33 cases (15%)
Diabetes	24 cases (11%)
Chronic hepatic diseases	11 cases (5%)
AIDS	11 cases (5%)
Collagen and vascular diseases	8 cases (3%)

Source: Adapted with permission from J. Rocourt and R. Brosch, *Human Listeriosis*—1990, WHO/HPP/FOS, © 1992, World Health Organization.

pressed individuals in a certain geographical area tends to reflect the characteristics of the local high-risk population.[78] Women who present with preterm labor should be suspected of being infected until proven otherwise. Recurrent cases of human listeriosis can occur and are thought to be due to treatment failure.[345] The major manifestations of the disease in nonperinatal cases are septicemia and/or meningitis. In the US *L. monocytogenes* is the fifth most common cause of bacterial meningitis and is the most common organism causing meningitis in patients with underlying malignancies. It is the third leading cause of bacterial neonatal meningitis in the United Kingdom (UK).[483] Unique features of listeria meningitis include

- common occurrence of tremor, grand mal, and focal motor seizures;
- direct invasion of the cerebral cortex;
- most commonly invading the brain stem (rhomboencephalitis);
- the percentage of neutrophils in the CSF is lower than in meningitis caused by other bacteria, and there is often a high monocyte count in cases of listerial meningoencephalitis; and
- in keeping with the organism's intracellular habitat (see Section 44.4), Gram-stained smears of the CSF usually are negative.[474]

The tropism of *L. monocytogenes* for the central nervous system (CNS) remains unexplained, although it is known that CNS cases are associated with a high mortality rate and neurological sequelae among survivors. Studies in Denmark and Australia also have pointed to the importance of *L. monocytogenes* as a nosocomial pathogen.[258,396] The differential diagnosis and treatment of *L. monocytogenes* infections are shown in Table 44–3.

44.3.2 Neonatal Listeriosis

L. monocytogenes is one of the few bacteria able to cross the placenta and gain direct access to the fetus. This property leads to the unique clinical syndrome of granulomatosis infantisepticum, which is fatal in 35% to 55% of cases in newborn infants.[475] The organism has been found in amniotic fluid at levels of 10^8 cfu ml^{-1} [88] and has been shown, using an *in vitro* uterine strip-model, to be possibly involved in causing premature labor contractions.[295] An interesting report also described a case of listeriosis in a full-term (39 weeks, 3 days) infant where the placenta became infected (ie, placental cultures positive, whitish nodules, and microabscesses present on macroscopic examination), but not the fetus. The infant, normal at birth, later developed severe neurological sequelae, possibly because of placental damage altering the utero-placental blood flow, thus leading to fetal hypoxia.[6]

Infection in pregnant women usually results in a mild flu-like illness. Although it can occur at any time, infection of the fetus usually occurs in the third trimester and can lead to abortion, stillbirth, or delivery of an acutely ill baby, although more commonly infection presents as preterm labor. Maternal listeriosis does not always result in infection of the fetus, as has been demonstrated in cases where only one of a set of twins became ill.[331] An interesting study showed that women who carry more than one child have a nearly 4-fold greater risk of acquiring listeriosis than pregnant women with singletons.[331] This risk of listeriosis in multiple-gestation pregnancies was also associated with age (ie, with women more than 35 years). Besides the well-known factors of reduced fetal and maternal immunity during pregnancy, in multiple-gestation pregnancies compared with single births, there may be increased production of hormones or other immunological effectors in the larger placenta.[331] Cases of first- and second-trimester listeriosis have occurred where treatment of the infected mothers led to the delivery of healthy babies.[169,176] Healthy pregnant women may be carriers of the organism and still give birth to a healthy child. Several cases of listeriosis in pregnant women with impaired immunity have led to the suggestion that in this group of individuals, listeriosis may be an important unrecognized disease.

Two clinical forms of neonatal listeriosis are known: early and late onset. In early-onset listeriosis

Table 44–3 Differential Diagnosis and Treatment of *Listeria monocytogenes* Infections

Category	*Differential Diagnosis*	*Treatment*
Pregnancy infections	Influenza Pyelonephritis Septic abortion	Ampicillin or penicillin
Granulomatosis infantiseptica	Neonatal sepsis Neonatal meningitis due to enteric bacilli or group B streptococcus	Ampicillin and gentamicin
Sepsis	Sepsis of unknown source due to a large variety of organisms	Ampicillin or penicillin
Meningoencephalitis	Psychiatric illness or metabolic encephalopathy Infection due to *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis,* or *Cryptococcus neoformans* in immunosuppressed patients or to group B streptococci or enteric bacilli in neonates	Ampicillin or penicillin, possibly with gentamicin intrathecally
Cerebritis	Brain abscess, tumor, stroke	Ampicillin or penicillin
Focal infections	Varies with site	Ampicillin or penicillin

Source: Adapted with permission from G.L. Armstrong, M.P. Doyle, and J.E. Bennett, *Principles and Practices of Infectious Diseases*, 4th ed., G.L. Mandell, J.E. Bennett, and R. Dolin, eds., p. 185, © 1994, Churchill Livingstone.

(0 to 2 days after birth) infection occurs in utero, while in late-onset cases (more than 5 days after birth), infants are either colonized at birth with a delayed onset of infection or disease is acquired by cross-infection from environmental sources. The majority of cases observed fall into the early-onset category. Most neonatal deaths from congenital listeriosis appear to be due to pneumonia and respiratory failure. It is not known why CNS infections, although common in nonperinatal cases, are rarely observed in pregnant women.[339]

Possible diagnostic features of neonatal listeriosis include bright green meconium, the presence of miliary granulomata and microabscesses in the placenta, and the presence of Gram-positive bacilli in clinical specimens. Early diagnosis and treatment are crucial to the survival of the infected neonate. Gram-staining of the gastric aspirate may be a reliable test for the early diagnosis of listeric infection. There is no definitive evidence that listeriosis is associated with repeated spontaneous abortions or infertility. Despite modern antibiotic therapy, neonatal listeriosis still has a mortality rate of about 35%.[142,339] Factors associated with a fatal outcome include low birthweight, early gestational age, and a long interval between symptom onset and delivery of antibiotics.

44.3.3 Noninvasive Listeriosis

Although noninvasive syndromes have been well characterized for some of the better-known foodborne pathogens such as *Clostridium perfringens* and *Bacillus cereus,* mild illness due to *L. monocytogenes* has not been well characterized. There are instances where only a mild, upper GI illness occurs after the ingestion of food containing the organism.[95,413,426] Such mild cases would go unnoticed unless they were part of a common-source outbreak. It is not known how many mild cases occur each year or what the ratio of noninvasive to invasive cases is.

44.3.4 Healthy Carriers

L. monocytogenes is a normal resident of the intestinal tract in humans and can be found in from 2% to 6% of samples analyzed from a variety of different populations, including pregnant women, healthy individuals, and those with various symptoms or underlying illnesses.[267,291,318] The reported incidence of the organism in feces depends on the methodology used and the number of fecal specimens submitted for analysis.[318] Carriage of the organism is more common, however, in sporadic cases and in household contacts of listeriosis patients than in the general population. Of 82 individuals from 28 households in which a case of listeriosis had occurred, 15 household contacts (18%) carried *L. monocytogenes* with the same serotype and enzyme type as the corresponding case-patient.[449] However, there was no significant difference in the presence of GI or other symptoms between carriers and noncarriers. Secondary cases of listeriosis occur rarely, and host susceptibility plays a crucial role in determining the outcome of the host's exposure to this organism. In sporadic cases, the proportion of excretors was high (21.6%), with levels of the organism reaching 10^4 organisms g^{-1} of feces.[256] Fecal excretion of the organism can last for at least 2 weeks. A study in the UK suggested that carriage may be seasonal, being more common in July and August. Studies with pregnant women[212,267] and renal transplant patients[318] demonstrated that even in high-risk groups, fecal carriage does not necessarily lead to clinical illness; thus, fecal screening for this organism as an indication of recent or current infection, or analysis of fecal samples during pregnancy to indicate a risk of listeriosis, is not warranted.[212]

44.3.5 Serotypes Involved in Listeriosis

Although human listeriosis can be caused by all 13 serotypes of *L. monocytogenes,* the majority of cases are caused by only three serotypes: 1/2a, 1/2b, and 4b. Geographic differences in serotype prevalence are seen: although 4b predominates in many European countries, there appears to be an even distribution of serotypes 1/2a, 1/2b, and 4b in cases of listeriosis in Canada and the US and a predominance of serotypes 1/2a and 1/2b in cases in countries such as Australia and Hungary.[430] The reasons for the differences in serotype distribution are unknown. It is unclear why only three serotypes cause most of the illnesses, although it has been suggested that these serotypes are more pathogenic than the others. Most of the large listeriosis outbreaks have involved a limited number of clones of serotype 4b, leading to the hypothesis that these constitute "hot" clones that are more virulent than other strains.

44.3.6 Strain Typing

Because most listeriosis cases are caused by only three serotypes, serotyping is of limited value in epidemiological investigations. Phenotypic methods such as biotyping, phage-typing, bacteriocin-typing, and MEE are used to differentiate strains. Many of these methods need specialized reagents, and only a few reference laboratories around the world can perform them reliably. Other problems with some phenotypic methods include nontypeability of strains and nonreproducibility. Phage typing is still used widely to type strains of *L. monocytogenes* and has proven to be useful in the screening of large numbers of strains in outbreak situations.[252] In studies of nearly 100 strains of *L. monocytogenes*, phage typing was the most discriminatory of four typing methods evaluated, followed by restriction endonuclease analysis (REA) typing, MEE, and ribotyping.[377,379] There have been recent attempts to optimize the current internationally accepted phage typing system for *L. monocytogenes.*[327] In general, phage typing is not considered to be as reproducible or discriminating as most molecular typing methods except for ribotyping.[21,45] However, phage typing is very suitable for mass screening of strains, when cost and complexity of handling are taken into account. Other typing methods for *L. monocytogenes* that are useful for mass screening include esterase,[195] monocin,[21] and lectin[133] typing.

In using MEE for typing, bacterial strains are differentiated according to the variation in electrophoretic mobility of a variety of enzymes. The diversity revealed allows differentiation of a number of electrophoretic types (ETs) within a single microbial species, with the combination of all electromorphs obtained for a strain being referred to as an ET. MEE has been applied successfully to *L. monocytogenes.*[39,44,223,380,400]

Some of the current genotypic methods used for typing strains of *L. monocytogenes* can be seen in Table 44–4. The same food sample may contain more than one clone or strain of *L. monocytogenes,*[102,309] and thus, depending on the reasons for typing, consideration must be given to the number of colonies isolated and/or tested from a selective plate.

Chromosomal DNA restriction analysis, REA, was one of the first chromosomal DNA-based typing methods used for *L. monocytogenes.*[523] In this method, chromosomal DNA is isolated, cut with a frequent-cutting restriction enzyme, and electrophoresed on agarose gel. The separated fragments, which usually range in size from 1,000 to 20,000 bp, are then visualized by staining with ethidium bromide. The complexity of the banding patterns obtained can limit the usefulness of the method because numerous bands are obtained when using a 4- or 6-base cutter against whole chromosomal DNA.

Table 44–4 Some Examples of Molecular Typing of *L. monocytogenes* by Genotypic Methods

Typing Method	*Number of Strains Analyzed*	*Number of Profiles Obtained*	*Number of Primers or Enzyme Type Used*	*Comments*	*Reference*
RAPD	51	29, 31	2	15 isolates from two outbreaks, plus 36 epidemiologically unrelated isolates	313
RAPD	115	11, 16	2	Isolates from a raw shrimp processing plant	102
RAPD	289	18	1	Isolates from poultry-processing environment: 64% of all strains displayed a single RAPD profile	294
RAPD	95	71	6	Classified bands as major or minor	384
RAPD	52	23, 19, 26	3		143
PFGE	115	13, 15	*Sma*I, *Apa*I	Isolates from a raw shrimp processing plant	102
PFGE	51	22, 26	*Apa*I, *Sma*I	Clinical isolates	313
PFGE	176	63, 72	*Asc*I, *Apa*I	CHEF system used	56
PFGE	35	17, 18, 15	*Apa*I, *Sma*I, *Not*I	FIGE system used	60
Ribotyping	94	14	*Eco*RI, *Hind*III	Used cloned rDNA probe from *Bacillus subtilis*	251
Ribotyping	305	28	*Eco*RI	Used *E. coli* rrnB operon (plasmid PKK 3535)	209
PCR-ribotyping	41	6 (serotype 4b) 8 (serotype 1/2a) 11 (serotype 1/2b)	1 pair		472
Southern hybridization fingerprinting	862	32	*Nci*I	Used probes λNS32 and 35, selected from a *L. monocytogenes* gene library	425
Restriction endonuclease analysis	120	10	*Eco*RI	All 4b isolates	376

PCR, Polymerase chain reaction; CHEF, Contour-clamped homogenous electric field gel electrophoresis; FIGE, Field-immersion gel electrophoresis.

Ribotyping refers to the use of DNA probes to recognize ribosomal genes. Ribosomal RNA (rRNA), present in all bacteria, makes up approximately 82% of the total RNA in a typical (ie, *E. coli*) bacterial cell and is composed of three molecular species: 23S, 16S, and 5S rRNA. The genes coding for rRNA are highly conserved (ie, the rRNA genes in many different bacteria are very similar). While most bacterial genes are present in only one copy, rRNA (or *rrn*) operons can be present in from 2 to 11 copies per bacterial cell, with *L. monocytogenes* containing 5 copies.[238] The more copies of a *rrn* operon present in a particular bacterial species, the more discriminatory ribotyping will be. In practice, the bacterial chromosomal DNA is isolated, and a restriction enzyme is used to cut the DNA into many small fragments. Once the restricted DNA has been electrophoresed on agarose gel, it is transferred either electrophoretically or by capillary action onto nylon or nitrocellulose membrane and then probed with labeled probes containing (usually *E. coli*) rRNA sequences. Besides good reproducibility, ribotyping has the advantage that universal probes can be used because of the similarity of ribosomal genes. The method has been automated using the Riboprinter™ Microbial Characterization System (E.I. Dupont, Wilmington, DE). This system divides the whole process into seven stages, and can ribotype 32 strains in four batches within 16 hours (Technical Literature, Dupont Riboprint™, E.I. Dupont). Graves et al.[209] examined 305 *L. monocytogenes* isolates from clinical and nonclinical sources and found that overall, MEE typing was more discriminating than ribotyping, but that in some cases, ribotyping was able to further subtype isolates that fell within the same ET. Ribotyping has also been found to be less discriminating than PFGE or RAPD analysis.[313]

Besides traditional ribotyping methods, there are different variations of the method that can fall under the general heading of southern hybridization fingerprinting. In this technique, instead of using rRNA as a probe, any other labeled probe that can identify a complementary DNA molecule among other DNA fragments in a gel is used. For example, probes containing toxin genes, insertion sequences (also referred to as insertion sequence or IS typing), or bacteriophage DNA can be used.[415]

PFGE typing is a method that tries to eliminate some of the problems that occur with REA, where hundreds of different bands can appear. PFGE uses restriction enzymes that are infrequent cutters, giving a small number (5 to 20) of very large, well-resolved bands, typically ranging in size from 10 to 800 kb. PFGE has been used to compare *L. monocytogenes* strains involved in outbreaks,[61,252] as well as to discern genome size differences in the different serovars of *L. monocytogenes.*[73] Genomic divisions delineated through PFGE, as well as through MEE, correlate directly with flagellar H antigen types and, therefore, to the serovars of listeriae.[39,56]

RAPD typing is one of the newer genotypic methods based on the polymerase chain reaction (PCR) (see Chapter 64). The use of PCR for RAPD differs slightly from the "normal" PCR reaction in two major respects: (1) the primers used are short (only 9 or 10 mers or nucleotides) and the sequences are chosen at random, thus no prior knowledge of the target DNA sequence is needed, and (2) the annealing temperature for RAPD is lower than for a normal PCR, and a single primer with no known homology to a genome can anneal at sites for which the match is imperfect. Purified DNA can be used for RAPD, but whole bacterial cells or supernatants from boiled cells are more convenient and equally effective.[335,336] Although there are advantages to RAPD typing, the major concern has been with reproducibility. Recent work, however, with *L. monocytogenes* (Table 44–4) has shown that the method can be reproducible under carefully controlled conditions. The method has been used successfully for strain discrimination in sporadic cases or outbreaks of listeriosis and to map the distribution of the organism in fish,[102] dairy,[514] and poultry processing plants.[294] A relatively new genotypic typing method also based on the PCR has been described.[257,288] The method takes advantage of the heterogeneity found in the spacer regions that exist between rRNA genes in microorganisms, ie, for a particular strain, multiple bands obtained after amplification represent spacer regions of various length in different ribosomal operons. In this method, however, instead of using arbitrary primers as in RAPD, one has to have some knowledge of the rRNA sequence. This technique has promise as an alternative to traditional *L. monocytogenes* typing methods.[288,472] The advantages of PCR ribotyping are that universal bacterial primers can be used; PCR-ribotyping produces stable, easily detectable amplification patterns in a rapid manner; and it has the potential to be widely useful in molecular epidemiology.[288] Its main disadvantage is its lower discriminatory power compared with PFGE, and possibly RAPD, typing.

Genotypic typing methods have increased greatly our ability to discriminate between strains of *L. monocytogenes.* None of the present subtyping methods can be used, however, to identify which strains of the organism are nonpathogenic, or less virulent.[342]

44.4 VIRULENCE

44.4.1 Virulence Factors

L. monocytogenes has an unusual life cycle. By a complicated series of host-pathogen interactions, the organism is able to enter a host cell, move across the cell cytoplasm, and enter neighboring host cells without becoming exposed to the extracellular environment (Figure 44–2).[456,474] Recently, many of the virulence factors and their respective genes have been identified, with eight of these genes grouped in a polycistronic cluster on the chromosome under the regulation of the *prfA* gene product (Figure 44–2).[75] The pathogenicity of the organism is thus multifactorial, resulting from the activity of products of at least nine genes.[342]

In foodborne infections, *L. monocytogenes* is consumed in contaminated food and possibly enters the host via intestinal epithelial cells[268] or the M cells of Peyer's patches. The bacteria then are internalized within a membrane-bound phagosome and transported to the liver, where they enter the hepatocytes by an induced phagocytosis.[484] Leukocytes rapidly are marshaled to the foci of infection, where they appear to cause the destruction of the infected hepatocytes.[84] The bacteria then are released into the extracellular space, where some are ingested and destroyed by phagocytes. Two surface proteins, internalin and p60, have been implicated in the induced internalization of *L. monocytogenes* by nonprofessional phagocytes (Figure 44–2).[180,286] Internalin was identified in screening a library of Tn*1545* mutants with the finding that, in mutants defective for entry into Caco-2 intestinal epithelial cells, the transposon had inserted into a region upstream from two open reading frames (ORF), *inlA* and *inlB*.[180] Internalin, the product of *inlA*, is a 90-kDa protein with a signal sequence, two regions of repeats, and a COOH-terminal hydrophobic region that is likely to be a membrane anchor.[123,180] Region A consists of 15 highly conserved, leucine-rich repeats (LRR). The receptor for internalin has been identified as E-cadherin, a surface protein involved in cell-to-cell binding.[355] InlB, the protein encoded by *inlB*, is a 630-amino acid protein containing 8 LRR with homology to those of internalin, and no membrane anchor.[123] *InlA* and *inlB* seem to be part of a multigene family, as several sequences showing homology are present on the chromosome.[123,180] A novel 30-kDa protein has been identified as a member of this multigene family and has been designated internalin-related protein.[305] Perhaps this *inl* repertoire encodes surface proteins with different specificities, as it has been shown that *inlA* and *inlB* gene products are essential for entry into epithelial cells and hepatocytes, respectively.[124] However, an additional *inlAB*-independent mechanism may exist, though an *inlAB* mutant was 50- to 100-fold less invasive for Caco-2 cells than the parent strain, because the mutant was still able to enter the cells.[179] Internalin is expressed more strongly at 37° C than at 20° C, and is under the control of the regulator gene *prfA*, though it is on a different region of the chromosome from the *prfA* regulon (Figure 44–2).

Spontaneous rough mutants of *L. monocytogenes*, that produce reduced levels of a 60-kDa protein called p60, form long chains and show reduced adherence, invasiveness, and virulence.[286,287] Deletions of the gene coding for p60, *iap* (invasion-associated protein), are lethal.[286] The *iap* gene encodes a 484-amino acid protein with a signal sequence but no membrane anchor, which is likely to be a mureine hydrolase involved in septum formation.[529] Its exact role in invasion is unclear,[227] but perhaps the lowered invasiveness of the rough mutants can be explained by the long chains in which they are associated.

Part of the process of internalization involves the ability of these bacteria to react with host cell–receptor molecules to induce their own phagocytosis. Two host protein kinases become tyrosine-phosphorylated during invasion of the host cell by *L. monocytogenes*. Inhibition of this process by protein tyrosine kinase inhibitors blocks *L. monocytogenes'* entry into host epithelial cells, suggesting that *L. monocytogenes* activates a protein kinase during invasion.[485,509]

Once internalized within a phagosome, which is a toxic environment for bacteria, *L. monocytogenes* lyses the membrane of the vacuole after about 30 minutes and gains access to the cytoplasm, where it can multiply. Two proteins, listeriolysin O (LLO), the first identified listerial virulence factor,[350] and a phosphatidylinositol-phospholipase C (PI-PLC)[202,469] are involved in this lysis. LLO, a 58-kDa protein, is a member of the family of pore-forming, thiol-activated cytolysins, of which streptolysin O is the prototype.[350] It is encoded by *hly*, the third gene in the *prfA* regulon. When this gene was cloned into *Bacillus subtilis*, it enabled that bacterium, after internalization within a macrophage, to disrupt the phagosomal membrane and grow within the mac-

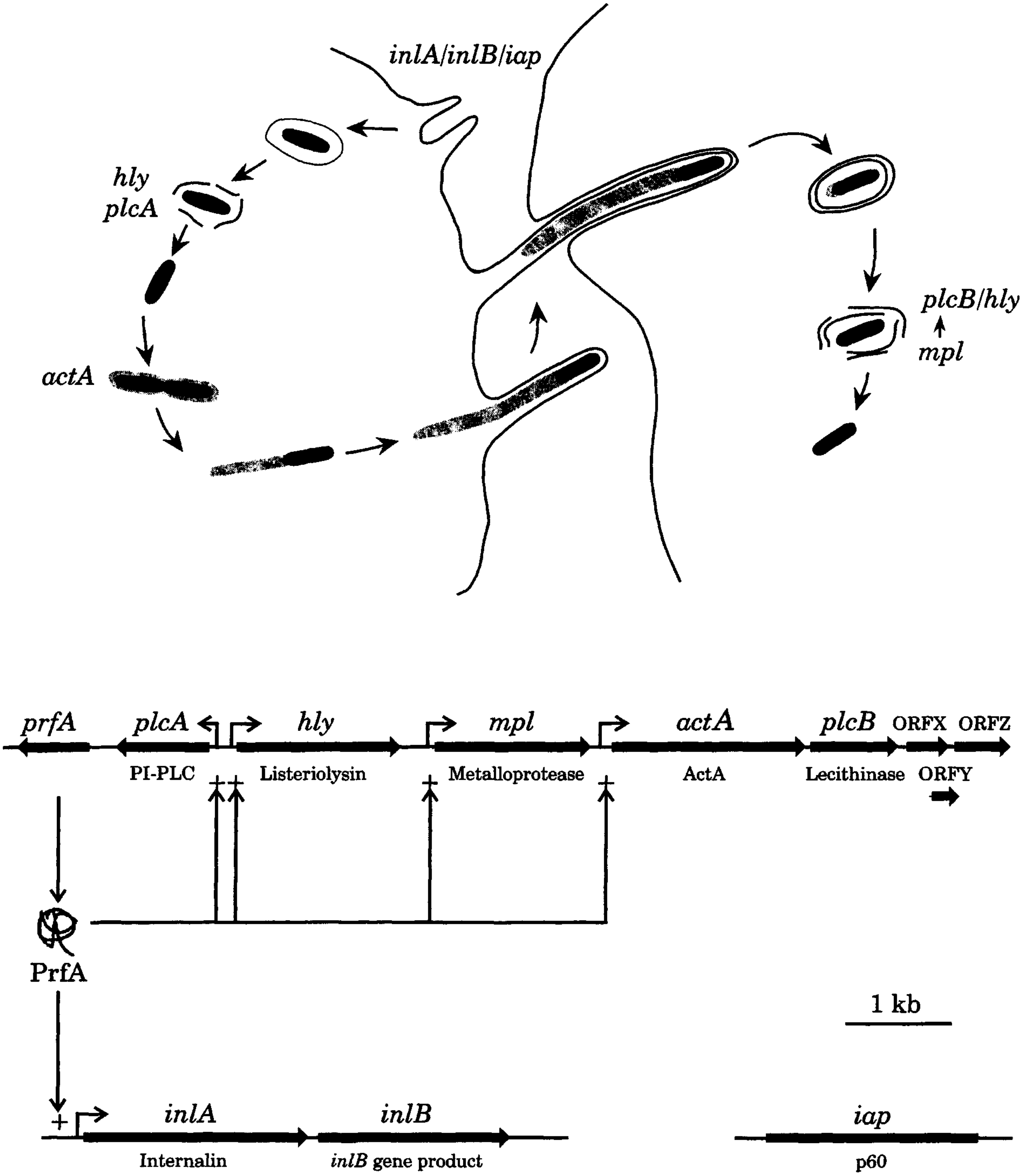


Figure 44–2 The Infectious Process and Virulence Factors of *Listeria monocytogenes*. A diagram of the infectious process is shown, together with the genes required at each step of the process. The arrangement of the various virulence factor genes on the chromosome is shown beneath, including the interaction of PrfA with its target sites. *Source:* Adapted with permission from S. Dramsi, C. Kocks, C. Forestier, and P. Cossart, Internalin-mediated Invasion of Epithelial Cells by *Listeria Monocytogenes* is Regulated by the Bacterial Growth State, Temperature and the Pleiotropic Activator *prfA*., *Molecular Microbiology*, Vol. 9, pp. 931–941, © 1993, Blackwell Science, Ltd.; and B. Sheehan, C. Kocks, S. Dramsi, et al., Molecular and Genetic Determinants of the *Listeria Monocytogenes* Infectious Process, *Current Topics in Microbiology and Immunity*, Vol. 192, pp. 187–216, © 1994. Springer-Verlag.

rophage cytoplasm.[40] Unlike other thiol-activated cytolysins that act extracellularly, LLO has a pH optimum of 5 and is able to exert its lytic activity within the acidic environment of the phagosome.[456]

PI-PLC is encoded by *plcA*, a gene upstream from *hly* in the *prfA* regulon (Figure 44–2).[297,352] PI-PLC is a 37-kDa phospholipase specific for PI and is active on a PI-glycan-anchored protein.[202] Transposon mutations in *plcA* result in a loss of virulence in mice, but this is probably due to a polar effect on the regulatory gene *prfA*, which is downstream from *plcA*.[66] The product of this gene plays a significant role in mediating escape of *L. monocytogenes* cells from phagosomes after internalization.[66,408]

For movement across the cell and into neighboring cells, *L. monocytogenes* appropriates the host cell actin machinery.[86,94,492,494] The ability to spread within host tissues by direct cell-to-cell spreading constitutes an essential feature of its virulence.[443] Three genes, *mpl*, *actA*, and *plcB*, have been implicated in this process.[113] These genes, together with three small ORFs downstream from *plcB*, form the lecithinase operon. ActA, encoded by *actA*, is a surface protein with an apparent molecular weight of 90 kDa and with a membrane anchor at its COOH-terminal end.[113,284] The amino-terminal end of ActA is critical for actin assembly and bacterial movement.[293,468] ActA conferred actin-based motility on the nonpathogen *L. innocua*.[285] ActA recruits the host cell actin to assemble continuously at one pole of the bacterial cell surface, forming an actin tail up to 5 μm long, which propels the bacterium across the cytoplasm, pushing on the host cell membrane and forming a protrusion, which can be ingested by an adjacent cell (Figure 44–2).[495,496]

Actin is the most abundant protein in the mammalian cytoplasm, existing either as ADP- and ATP-actin monomers or as filaments formed by head-to-tail polymerization of the asymmetric monomers. The first step in the nucleation process, which seems to be under ActA control,[173] involves the combination of three ATP-actin monomers to form an unstable trimer. This elongates in a polar action, by the addition of monomers more rapidly to the end of the asymmetric actin tail that is attached to the bacterium.[87] The host cell protein, profilin, binds to monomeric ADP-actin, distorting its structure and allowing the exchange of ATP for ADP,[283,493] thereby promoting actin filament assembly. Profilin is the only actin-regulatory protein that binds to polyproline. Each molecule of ActA bacterial protein contains four proline-rich sites that bind the host cell protein, vasodilator-stimulated phosphoprotein (VASP).[404,406] Each VASP monomer contains four polyproline-rich sites, each with the potential to bind profilin. Because VASP exists as a tetramer in the host cell, each ActA molecule can therefore attract up to 64 molecules of profilin, amplifying the ATP/ADP exchange in the promotion of actin filament assembly. The elongation of the filament is promoted, supplying the motive force for the migration of the bacterial cell through the cytoplasm.[76,468] A number of additional host cell proteins regulate the filament assembly, including filament-capping proteins, cross-linking, and bundling proteins.

On arrival at the plasma membrane of the host cell, the bacteria induce the formation of pseudopod-like membrane protrusions. Contact between these protrusions and the plasma membrane of adjacent cells results in the internalization of the protrusion containing a *L. monocytogenes* cell, which is now surrounded by a double layer of plasma membrane (Figure 44–2). Lysis of these vacuoles involves a broad-range lecithinase or phospholipase C (PLC), encoded by *plcB*.[189,420,469,507] PLC is a 29-kDa protein, synthesized as a precursor, which is then cleaved by a zinc metalloprotease,[409] which is encoded by the *mpl* gene.[112,354] LLO may also contribute to the lysis of the double membrane vacuole, though in LLO⁻ mutants, the broad-range PLC and the metalloprotease were sufficient for lysis in Henle 407 cells.[328]

There have been no reports of listerial virulence factors that have systemic effects, except for that of a cholera toxin-like protein encoded by a gene that has been sequenced.[183]

44.4.2 Regulation of Virulence Factors

Complementation analysis and studies of transposon or insertion mutants demonstrated that *prfA* is a pleiotropic activator of virulence gene expression.[122,353] It regulated a polycistron containing eight genes,[75,200] as well as the *inl* multigene family located elsewhere on the chromosome (Figure 44–2). PrfA, its gene product, is a 27-kDa protein that activates transcription from the *plcA, hly, mpl,* and *actA* promoters, as well as the *inlAB* promoter.[171] Activation was most efficient at the divergently transcribed *plcA* and *hly* promoters; the activation of *mpl* and *actA* was considerably weaker.[457] Transcription of *prfA* does not occur below 30° C.[298] PrfA shows similarity to the *E. coli* cAMP receptor protein and is a site-specific DNA-binding protein.[172]

Apparently, the PrfA protein acts through direct binding to specific sites in front of the respective gene promoters,[46] because on gel electrophoresis it retards the mobility of DNA fragments containing the 14 bp palindromic sequence located between the divergent *hly* and *plcA* promoters. Similar palindromic sequences are found in the –35 regions of *mpl*,[351] *actA*,[507] and the internalin locus promoters.[123] *InlA* and *inlB* are transcribed in their operon and individually by both PrfA-dependent and PrfA-independent mechanisms.[304]

The regulation of *prfA* expression is complex. The gene is not constitutive, as two peaks of *prfA* transcription occur during the growth cycle *in vitro*.[353] The first occurs in early exponential phase when *prfA* is cotranscribed with *plcA* from the 3' end of the *plcA* gene. The second occurs at the end of exponential growth, is monocistronic, and originates immediately 5' to the *prfA* gene. The primary promoter for the cotranscription of the *plc-prfA* operon is regulated by *prfA*; thus, *prfA* autoregulates its own synthesis.[172] Dual promoters have been demonstrated immediately upstream of *prfA* coding sequences.[170] *PrfA* was among five genes preferentially expressed when *L. monocytogenes* was in an intracellular environment in infected macrophages.[278]

44.4.3 Host Cell Protective Mechanisms

Although it was once thought that immunity to *L. monocytogenes* could be explained entirely by the generation of activated macrophages, it is now clear that the immune response to the organism encompasses both an innate and an acquired response.

Macrophages become activated in the defense of the mammalian host against intracellular listerial infections with the formation of reactive oxygen and nitrogen intermediates such as superoxide.[228] The bacterial enzyme, superoxide dismutase (SOD), converts superoxide into hydrogen peroxide, which is further metabolized by catalase, thus counteracting the defense mechanisms of activated macrophages. The SOD from *L. monocytogenes* has been sequenced and shows close similarity to Mn^{++}-containing SODs from other organisms.[54]

Acquired immunity to listerial infections is cellular in nature with antibodies playing no role because the bacteria never confront the humoral immune system. Immunity to *L. monocytogenes* is restricted largely to major histocompatibility complex class I antigens, with the processing of the antigens in the cytoplasmic environment, and their presentation to $CD8^+$ T cells.[407] Thus the organism must lyse the phagosome and enter the cytoplasm for the induction of immunity.

The question of the minimum infectious dose (MID) of *L. monocytogenes* for humans is a vexing one. There is some indication of strain dependency because many of the larger foodborne outbreaks have been due to serotype 4b. The immunological status of the host seems to be a critical factor because few cases of listeriosis are reported in adult, immunocompetent persons. Oral challenge of *Cynomologous* monkeys with 10^5, 10^7, and 10^9 *L. monocytogenes* showed that only those receiving 10^9 bacteria developed fever, septicemia, loss of appetite, irritability, and occasional diarrhea.[151] All listeriosis cases published from foodborne outbreaks where the implicated food has been identified were caused by foods containing ≥100 cfu *L. monocytogenes* g^{-1}.

44.5 INCIDENCE OF *L. MONOCYTOGENES* IN FOOD AND THE ENVIRONMENT

44.5.1 Dairy Products

Since dairy products were one of the first food groups reported to be involved in outbreaks of listeriosis, extensive surveys have been made of the incidence of *Listeria* on the farm and in food processing environments, as well as studies on the incidence, survival, and growth of the organism in dairy products. *L. monocytogenes* is endogenous to the farm environment, where it can be found in water, fecal material, soil, and cow's hair. The organism can be transmitted to cows via feeds such as improperly fermented silage, hay, and oatmeal as well as by straw and fecal contamination (Figure 44–1).[214,244,501] The relationship between silage feeding and animal listeriosis has been known for years.[210] The survival of the organism in silage depends on the complex interaction between dry matter, O_2 tension, grass, and microbiological quality. In general, the organism will survive and grow well in silage with an O_2 concentration of ≥ 1% and a pH > 5.0.[115,399,501] However, the organism has been found in silage ranging in pH from 3.8 to 5.2.[422] In addition, mastitis occurs in animals, with consequent chronic shedding of the organism for a number of years.[116,150] The organism has been recovered from mastitic animals at levels ranging from 10^3 to 10^4 cfu ml^{-1} of milk. There is a wide range in the reported incidence of the organism in raw milk (Table 44–5). In a large case-control study involving 128 dairy farms, factors associated with raw milk contamination by *L. monocytogenes* included poor quality silage, infrequent cleaning of the exercise area, poor cow cleanliness, insufficient lighting of milking barns and parlors, and incorrect disinfection of towels between milkings.[441] Samples obtained from factory silos or large processing centers have a much higher incidence of contamination than do bulk tank samples (Table 44–5). This is probably due to the pooling of contaminated milk samples. The levels of the organism in raw milk are usually less than 1 cfu ml^{-1}, but can range up to 62 cfu ml^{-1}.[158,383] Although O'Donnell[383] and Rea et al.[422] found a marked seasonal trend, with the highest incidence of *Listeria* in raw milk in the winter months when the cows were housed indoors, other investigators have found a higher incidence in the summer.[148,157] O'Donnell's work showed that as a percentage of total *Listeria* isolated, *L. monocytogenes* was the dominant species isolated during the summer months. The organism grows well in both raw and pasteurized milk, with reported generation times at 10° C of about 10 hours.[147,330]

Much evidence suggests that environmental contamination, in particular postprocessing contamination, is a major source of contamination of food (Figure 44–1). The organism is prevalent in the environment and on equipment in dairy processing operations.[67,77,279,411,412] Sites typically positive for *L. monocytogenes* include drains, conveyor belts, floor mats and foot baths, coolers, freezers, areas underneath equipment and associated with case washers, raw milk-associated areas, cheese-ripening rooms, and cheese washing brushes. A large study[411] of the incidence of *Listeria* in the environment of 30 dairy processing plants, either with or without an onsite dairy farm, found that 57.9% and 38.5%, respectively, of the sites tested were positive for *Listeria*. No one area appeared to be more highly contaminated than any other. The environment of a dairy operation can be more contaminated than the equipment within the same plant (ie, 6 of 21 plants testing positive for equipment versus 19 of 21 plants having positive environmental samples).[412] The authors suggested that even when the organism is present in the environment of a particular plant, this will not automatically lead to prolonged contamination of the equipment.[412]

Table 44–5 Incidence of *L. monocytogenes* in Raw Milk

Country	*Number of Samples*	*Number (%)* L. monocytogenes *Positive*	*Major Serotype Involved (%)*	*Reference*
Brazil	220	21 (9.5)	NK[a]	364
Canada	315	17 (5.4)	1 (82.4)	466
Canada	455	6 (1.3)	NK	148
Hungary	80	3 (3.8)	1 (100)	431a
Ireland	589	29 (4.9)	NK	422
Ireland	113	6 (5.3)	1 (89)	222
	63[b]	21 (33.3)		
Italy	40	0 (0.0)		332
Morocco	30	3 (10)	NK	130
New Zealand	71	0 (0)		479
Scotland	540	14 (2.6)	1 (76.9)	157
Spain	95	43 (45.3)	NK	114
Switzerland	317	4 (1.3)	NK	53
Transvaal,	961	50 (5.2)	4 (92.5)	528
South Africa	21[c]	17 (81.0)		
Turkey	77	14 (18.2)	NK	455
United Kingdom	361	13 (3.6)	1/2 (85.0)	213
United Kingdom	480[d]	4 (0.8)	1/2 (100)	213
United Kingdom	2009	102 (5.1)		383
United States	200	8 (4.0)	1 (71.4)	302
United States	650	27 (4.2)	1 (61.5)	315

[a] NK, not known.
[b] Samples obtained from large processing centers.
[c] Samples obtained from dairy factory silos.
[d] Goat milk.

44.5.2 Meat and Poultry Products

Contamination of meat products by *L. monocytogenes* can originate from the processing environment of the chilling or cutting room,[405,506] hands,[275] and equipment.[294] Additional major points of contamination include contact of cooked products with soiled surfaces, cross-contamination between raw and cooked channels, and the inadequacy of cleaning and disinfection procedures.[440] In a study that examined product and environmental contamination of six delicatessen plants in France, before disinfection transport belts, tables, gloves, hands, meat and jelly containers, and knives were the most highly contaminated, while after disinfection meat containers, knives, and caddies were the most highly contaminated.[440] In cattle and lamb, animal hides and pelts were more important sources of *Listeria* in the carcass than fecal contamination,[316] while in pigs, fecal contamination was the most important source of carcass contamination.[5]

L. monocytogenes can be isolated both at the abattoir[307,316] and at the retail level,[240a,294] and a wide variety of meats have been found to be contaminated with *L. monocytogenes* (Table 44–6).[142,261] The reported incidence of contamination varies greatly, due largely to methodology differences including such factors as the method used, sample size, the number of single colony isolates taken to confirm the presence of hemolytic colonies, and whether samples were purchased from retail or commercial outlets. Survey data show *L. monocytogenes* to be present in a wide variety of raw and ready-to-eat (RTE) meats (Table 44–6). As well as in North America and Europe, *L. monocytogenes* has been isolated from meat and meat products in South Africa,[513] Trinidad,[5] Japan,[438] China,[520] Malaysia,[13] and New Zealand.[405] Most of the observed contamination is on the surface; however, *L. monocytogenes* was found in the interior muscle cores of 5 of 110 total samples of beef, pork, and lamb roasts.[261] These organisms were probably present in muscle at the time of slaughter. Serotype 1 is the most frequent serotype found in meats worldwide.[142]

Poultry can become contaminated with *Listeria* either environmentally during production or from healthy carrier chickens in the processing plant.[18,186] Carcass gut contents, food handlers, contaminated surfaces (eg, "rubber fingers"), scald tank water, and contaminated equipment[126,190] have been implicated as sources of *Listeria*. A limited number of *Listeria*-contaminated poultry carcasses may contaminate a larger number of carcasses in the chilling water.[167] Contamination of turkey carcasses occurred after defeathering during either evisceration or chilling. Main sources of *L. monocytogenes* contamination were insufficiently cleaned equipment and the hands and gloves of workers in the final process areas.[81] Some strains of *L. monocytogenes* appear capable of adapting to certain niches in poultry plants, persisting for long periods of time (up to 1 year), and causing cross-contamination.[294]

Chicken can be heavily contaminated with *L. monocytogenes*, and the incidence of *L. monocytogenes* can range from 12% to 92% in raw broilers,[142,239,261,307] to 12% to 27% in cooked and RTE poultry products, respectively.[192,274,424] Franco et al.[167] found drumstick meat and skin to be the most contaminated of the poultry pieces examined, ie, 95% of samples contained *Listeria* spp. The authors concluded that drumsticks were responsible for a large amount of the contamination of chicken carcasses, that most contaminated areas were in the final stages of meat processing, and that work surfaces in contact with meat were an important source of *Listeria* spp. Both raw and cooked poultry products were the foods of greatest concern in terms of the incidence and levels of *L. monocytogenes*.[319,525] A cooked poultry product was found to contain 1,200 cfu g^{-1} of *Listeria*, while two cooked turkey products contained $>10^4$ cfu g^{-1} of *L. monocytogenes*.[319,525] Such numbers in cooked products might be due to undercooking or to contamination and multiplication after cooking. One would expect to see sporadic cases of listeriosis as the result of random occurrence of undercooking (Section 44.6.2).

Few studies have examined the serotype predominance in chickens, but it appears that, as for beef, serotype 1 is the most prevalent. Bailey et al.[18] examined the factors influencing colonization of broiler chickens with *L. monocytogenes*. Although *L. monocytogenes*, orally inoculated, does not colonize chickens as easily as do salmonellae or *Campylobacter jejuni*, younger birds were more susceptible to colonization than older birds, and there was a dose-related colonization response.

Most studies have found *L. monocytogenes* in meats initially at very low levels (<100 cfu g^{-1}). For example, the organism was present in wiener packages at levels of 0.34 to 2.3 MPN ml^{-1} of exudate.[519] Villari et al.[511] found levels of the organism to be highest in poultry, followed by pork and then beef. During quantitative analysis, however, most investigators do not include a resuscitation step. When a resuscitation step was used, the levels of *Listeria* spp. from meat and fish products were up to 2.5 logs

Table 44–6 Incidence of *Listeria monocytogenes* (*Lm*) in Meats

Meat Type (Country)	*Incidence of* Listeria *spp. (%)*	*Incidence of* Lm *(%)*	*Numbers*	*Comments*	*Reference*
Retail frankfurters (US)	9/93[a] (10)	7/93 (7.5)	0.34 to 2.3 MPN ml^{-1} of exudate	*Lm* found in 6 of 20 brands tested	519
Retail vacuum-packaged processed meats (Australia)	93/175 (53)	78/175 (44.6)	3.1–3.3 x 10^4 cfu g^{-1} [b]	corned beef most highly contaminated	208
Retail luncheon meat and pâtés (Spain)	30/96 (31)	21/96 (21.9)	83% of samples <10 cfu g^{-1}		290
Frankfurters and RTE sliced meats (Denmark)	ND[c]	38/304 (12.5) 41/304 (13.5)	99% of samples <100 cfu g^{-1}	4 x 10^3 cfu g^{-1} found in a frankfurter sausage at expiration date	416
Fresh ground beef, fresh pork meat, and sausages (Italy)	176/490 (35.9)	65/490 (13.3)	<100 (91.6–92.6%) 100–1000 (3.7–5.6%) 1000–2000 (2.8–3.7%)		83
Processed meats (US)	ND	294[d]/20,296 (1.4)	ND		329
RTE meats (Northern Ireland)	285/5020 (5.7)	ND	ND	undercooked chicken appeared to present the greatest risk	525

[a] Total number of positives/total number of samples.

[b] Obtained from 2 of 50 corned-beef packs in which *Lm* were counted.

[c] ND, not done.

[d] Plants with positive *Lm*.

RTE, ready-to-eat

higher than without resuscitation, indicating the presence of large numbers of injured cells that were undetected in the absence of resuscitation.[459]

In addition to the sporadic cases of listeriosis involving meat products, there have been numerous recalls in the US, Canada, and the UK of meat products containing *L. monocytogenes*. The products recalled have included turkey franks, farm sausages, cooked ham, prepared hamburger sandwiches, hot dogs, chicken spread, vacuum-packaged sliced ham, sausage sandwiches, chicken, egg and ham salads, frozen heat-and-serve chili dog and chili and cheese dog sandwiches, and Cajun pork sausages. The total economic loss to the respective companies has run well into the millions of dollars.

Because of the widespread distribution of *L. monocytogenes* in and around meat processing plants, it is unlikely that *Listeria* can be eliminated from meat packing plant environments.[261,294,405] Proliferation of *L. monocytogenes* in food plant environments is promoted by high humidity and nutrient waste.[446] However, in some operations, meat carcasses can be prepared free of *L. monocytogenes*, and proper cleaning of processing equipment can largely prevent meat becoming contaminated with *Listeria*.[194,405]

44.5.3 Fish and Seafood

Water plays a major role in the cycle of infection by *Listeria*[107] and may be the original source of seafood contamination. There are a number of ways by which *Listeria* can enter into the water supply—fecal contamination from animals, release of sewage effluent, and runoff from agricultural land.[175] *L. monocytogenes* can be found mainly in freshwater and seawater from coastal areas.[26]

In a study of the incidence and sources of *L. monocytogenes* in cold-smoked salmon plants, the primary source of contamination was the surface of the fresh or frozen raw fish coming into the plant, and during the filleting, rinsing, and brining stages, the organism could be transferred to the exposed flesh.[128] As the product moved through the different processing areas, anything that came into contact with the exposed contaminated flesh (ie, personnel, equipment, work surfaces) was liable to become contaminated and then serve as a secondary source of contamination.[128] In a cold-smoked salmon slaughterhouse and processing plant, a low incidence of the organism was found in the slaughterhouse and in the slaughtered fish, but a high incidence was found in the smokehouse.[433] Molecular typing of isolates revealed that there was one predominant ET (ET-6) present in the smokehouse; this same ET was the only one recovered from the vacuum-packed (VP) smoked salmon.[433] Although it appeared that a reservoir of *L. monocytogenes* was established in the smokehouse, the original source of contamination was not identified (ie, strains isolated from seawater and fish were not ET-6). *L. monocytogenes* could be recovered from many sites in a raw shrimp processing plant from processing water, utensils, food handlers, floors, walls, doors, and drains.[102] The number of different strains recovered from shrimp in the processing area was high. Since there were many different sources of the organism in the shrimp processing plant, the authors concluded that the elimination of *L. monocytogenes* from the frozen finished product would be virtually impossible under the processing conditions existing in the plant. Of three Swiss rainbow trout fish farms and their affiliated smoking plants, one plant was much more highly contaminated than the other two, both in the plant environment and in the product.[255] This was attributed to the fact that this plant did not have a regular cleaning and disinfection schedule and the fish had not been denied feed before slaughter; the practice of denying feeding is designed to reduce fecal contamination in the slaughter area.

A number of studies examined the incidence of *L. monocytogenes* in seafood with the prevalence of the organism in fresh and frozen fish, processed seafood, shrimp, and shellfish being 0% to 50%, 0% to 26%, 0% to 20%, and 0% to 7.5%, respectively.[26] The incidence of *L. monocytogenes* in tropical fish and fish products, including dried-salted fish, appears to be low, although only limited data are available. The overall incidence of *L. monocytogenes* in RTE cold-smoked salmon and cooked fish products has been estimated at 6% to 36%.[26] Of 377 samples of smoked and marinated fish, 47 were positive for *L. monocytogenes*[253] and of 100 smoked samples, 24% were positive for the organism. Compared with cold-smoking, which has little or no effect on *L. monocytogenes*, hot-smoking fish can lead to up to a 6-log reduction in numbers of the organism.[254] However, hot-smoking fish can lead to higher levels of *L. monocytogenes* on the product after storage than cold-smoking because of the elimination of the background microflora. Although the incidence in seafoods can be fairly high (Table 44–7), usually very low numbers (<100 cfu g^{-1}) of the organism are found.[128,137,421] A study in the UK found *L.*

monocytogenes in 128 (3%) of 4,435 seafood samples, but in only 8 of these were levels higher than 10^2 cfu g^{-1}.[343] A significant association was found between the presence of *Listeria* and high viable counts. The seafoods with the highest incidence (7%) of contamination with the organism were smoked fish and fish pâtés.

Fish products, nevertheless, have been involved in both sporadic cases and outbreaks of listeriosis (Table 44–8). In addition, many fish products in North America including frozen cooked shrimp, canned frozen, fresh and imitation crab meat, smoked salmon, imitation scallops, frozen canned lobster, and surimi products have been found to be contaminated with *L. monocytogenes* and been recalled from the market.

44.5.4 Vegetables

L. monocytogenes is widely distributed on vegetation and probably contaminates it via many routes including soil, water, animal manure, decaying vegetation, and sewage effluents (see Figure 44–1). Although many types of vegetables have been analyzed for *L. monocytogenes*, only potatoes and radishes appear to be contaminated on a regular basis.[149,226,464] On fresh-cut vegetables, the incidence of the organism varies from 0 to 19%.[70] Low levels of *L. monocytogenes* (<200 cfu g^{-1}) have been found in positive samples.[508] As with most foods, serotype 1 appears to be the predominant serovar.

44.6 EPIDEMIOLOGY

44.6.1 Food-Related Outbreaks

The vehicles of infection by *L. monocytogenes* in both sporadic and epidemic listeriosis were not proven until the early 1980s, although transmission in milk was suspected and direct transmission from infected animals to farm workers and veterinarians producing cutaneous lesions was well documented. However, an outbreak with 41 cases (34 perinatal, 7 adult) (Table 44–8) occurred in the Maritime Provinces of Canada between March and September 1981.[444] Of the 34 perinatal cases, there were 9 stillbirths, 23 live births of ill infants with a subsequent 27% mortality rate, and 2 live births of well infants. The adult mortality rate was 28.6%. During the epidemiological studies, an elderly individual was admitted to the hospital for what turned out to be a case of listeriosis. Because this was a frugal individual, leftovers remained in his refrigerator. Examination of all suspect foods revealed the presence of *L. monocytogenes* type 4b, the epidemic strain, in a packaged coleslaw mix that contained no dressing. Coleslaw was then included as a food item in the second food questionnaire, and the rest of the puzzle solved. The coleslaw mix had been prepared by a regional manufacturer, and distribution was confined to the Maritime Provinces. Environmental cultures taken at the implicated plant failed to reveal *L. monocytogenes*. A review of the sources of

Table 44–7 Incidence of *L. monocytogenes* (Lm) in Raw and Processed Seafoods

Product	*Total Number of Samples*	Listeria *spp. Positive (%)*	Listeria monocytogenes *Positive (%)*	*Reference*
Ceviche	32	24 (75)	3 (9)	174
Fresh shrimp	74	ND[b]	8 (11)	363
Fish and fish products	91	25 (27)	8 (9)	220
Seafood salads	37	12 (32)	6 (16)	220
Cooked blue crab	126	13 (10)	10[a] (7.9)	421
Cold-smoked salmon	61	ND	48[c] (78.7)	128
Slaughtered fish	50	1 (2)	0 (0)	433
Smoked salmon (VP)	65	7 (11)	7 (11)	433
Hot-smoked fish	142	36 (25.4)	12[d]/258 (4.7)	109
Cold-smoked fish	116	7 (6.0)	ND	109
Fish and shrimp	102	11 (10.8)	2 (2.0)	4
Fresh shrimp	178	ND	31 (17.4)	102

[a] Most samples contained <100 MPN g^{-1}. One sample contained 1,100 MPN g^{-1}.

[b] ND, Not done.

[c] Overall mean levels were 6.2.

[d] Only isolated from cod (11/60 samples) and herring (1/58 samples).

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Table 44–8 Outbreaks of Foodborne Illness Due to *Listeria monocytogenes*

Location (Year)	*Number of Cases (Deaths)*	*Perinatal/ Nonperinatal*	*Foods Associated*	*Serotype*	*Reference*
Boston, US (1979)	20 (5)[a]	0/20	raw celery, tomatoes, lettuce[b]	4b	229
New Zealand (1980)	29 (9)	22/7	shellfish, raw fish[b]	most cases 1/2b	301
Maritime Provinces, Canada (1981)	41 (17)	34/7	coleslaw mix	4b	444
Massachusetts, US (1983)	49 (14)	7/42	pasteurized milk[b]	4b	161
California, US (1985)	142 (48)	93/49	Jalisco cheese	4b	306
Switzerland (1983–1987)	122 (31)	65/57	raw milk cheese	4b	41
Philadelphia, US (1986–1987)	36 (16)	4/32	ice cream, salami[b]	4b,1/2a,1/2b,3b	451
Connecticut, US (1989)	9 (1)	2/7	shrimp[b]	4b	
United Kingdom (1987–1989)	355 (94)[c]	185/129[d]	pâté[b]	4b;4b(x)	426
West Australia (1990)	11[e] (6)	11/0	pâté[b]	1/2a	345
Tasmania (1991)	4	0/4	smoked mussels	1/2	277
New Zealand (1992)	4 (2)	4/0	smoked mussels	1/2	359
France (1993)	39 (12)	31/8	pork rillettes (pâté)	4b	19
France (1992)	279 (85)	92/187	pork tongue in jelly	4b	204
Italy (1993)	23 (0)	0/23	rice salad[b]	1/2b	205
Texas, US (1993–1994)	8 (1)[g]	4/4	frozen mixed vegetables[b]	4b	439
US[f] (1994)	45 (0)	1/44	(1%) chocolate milk	1/2b	101
France (1995)	20 (4)	11/9	raw milk soft cheese	4b	95, 413
Sweden (1994–1995)	6 (1)[h]	2/4	cold-smoked rainbow trout	4b	206
					127

[a] For two of these five deaths, an underlying disease, not listeriosis, was apparently the cause of death.
[b] Foods only epidemiologically linked.
[c] Outcome only known for 252 cases.
[d] Only 314 patients categorized.
[e] Includes two sets of twins.
[f] Outbreak was centered in Wisconsin.
[g] Spontaneous abortion.
[h] Not known if death due to listeriosis or leukemia.

raw vegetables for the plant identified a farmer who raised cabbage and kept a flock of sheep, of whom two had died of listeriosis, one in 1979 and one in March 1981. The cabbage was grown in fields fertilized by both composted and raw manure from this flock. Following the cabbage harvest each October, the crop was stored in a large cold-storage shed. Because *L. monocytogenes* is able to grow at low temperatures, the period of cold storage of the cabbages and the refrigeration of the coleslaw mix may have acted as a period of selective enrichment for the organism.

In the summer of 1983, an outbreak of listeriosis associated with *L. monocytogenes* type 4b occurred in Massachusetts (Table 44–8).[161] Of the 42 adult and 7 perinatal cases, 14 (29%) died. Case-control studies showed that the outbreak was strongly associated with drinking a specific brand of pasteurized milk. The case for an association with milk was strengthened by linkage of a specific phage type with the milk-associated disease. The milk associated with the outbreak came from a group of farms where bovine listeriosis was known to have occurred at the time of the outbreak. *L. monocytogenes* was isolated from raw milk at these farms. There was no evidence for improper pasteurization procedures at the plant, and this raised the question as to whether *L. monocytogenes* survived the pasteurization and whether its intracellular location gave it added protection.

The outbreak that raised listeriosis to a higher level of concern among food manufacturers and regulatory agencies occurred in California from January to August 1985 (Table 44–8).[306] Of the 142 reported cases, 93 were perinatal and 49 were adult, with 48 deaths (34% mortality rate): 30 fetuses and newborn infants and 18 nonpregnant adults. Among the 49 adult cases, 48 were immunosuppressed, elderly, or had a severe chronic illness. Case-control studies implicated Mexican-style soft cheese of a certain brand produced in plant A. The presence of *L. monocytogenes* type 4b, of the epidemic phage type, was confirmed in this cheese. Environmental samples taken from the plant producing the implicated cheese were positive for the epidemic phage-type organism. Although the pasteurizer was in good operational order, 9 of 80 cheese samples tested positive for phosphatase, suggesting a bypass of the pasteurizer (several occasions were documented of delivery of more raw milk than could be handled by the pasteurizer) or the introduction of raw milk after pasteurization. The factory was closed and the cheese of the implicated brand was recalled. *Listeria* spp. were not found in raw milk samples from the 27 dairy farms supplying the cheese plant; however, food products (Mexican-style sour cream and cottage cheese byproducts) from plant B, which was one of the major suppliers of raw milk to plant A, were also contaminated with the epidemic phage type. This is the first recorded listeriosis outbreak in which the food causing the epidemic was identified and recalled during the outbreak.

Another outbreak due to the consumption of soft cheese[41] occurred in western Switzerland (canton de Vaud). During 1983 to 1987, 122 listeriosis cases with 31 deaths were recorded (Table 44–8). More than 85% of the strains isolated during the epidemic period were of a similar serotype (4b) and phage type. Initially, a source of infection or route of spread could not be found; however, following the Jalisco cheese outbreak, an extensive survey of dairy products for *Listeria* spp. was carried out in Switzerland. When the isolates from this survey were sero- and phage-typed, only those recovered from the surface of Vacherin Mont d'Or soft cheese were identical to the outbreak strains. A follow-up investigation of the entire Vacherin production also turned up strains of a similar phage and serotype. Clinical cases were observed mainly during the winter period because the cheese was produced exclusively during the winter season and eaten in the area where the cases occurred. In late November 1987, all products were recalled from the market. This was an effective control procedure, in that the number of new cases in the area dropped substantially and no case due to an epidemic strain was recorded in 1988.[41] An examination of the 57 nonperinatal cases showed that there was an unusually high percentage (79%) of cases of CNS listeriosis in relatively young, previously healthy adults, with neurological sequelae developing in 30% of survivors, and mortality was positively associated with increased age and a clinical presentation of meningoencephalitis.[63]

A routine investigation of foods from a patient's refrigerator after a listeriosis case in the UK in 1989 led to finding a pâté sample that contained high levels of *L. monocytogenes*. This finding led to surveys, government health warnings to high-risk groups about pâté consumption, and a nationwide study to examine the relationship between human listeriosis and pâté consumption. During the 1989 survey, pâté samples from a single plant were more frequently contaminated with *L. monocytogenes* and at higher levels than those of other producers.[345] *L. monocytogenes* serotype 4b (phage type 6,7) and serotype 4b(X) accounted for 96% (48 of 50) of all isolates from the manufacturer's pâté, compared with 19% (6 of 31) of those from other producers in the 1989 survey, 4% (1 of 25) in the 1990 pâté survey, and rarely from other foods. The upsurge in cases of human listeriosis between 1987 and 1989 in the UK was due to these two serotypes, which accounted for 30% to 54% of the annual totals of cases. The two strains were much less common both before 1987 and after mid-1989.[345] Patients infected with the epidemic strains were significantly more likely to have eaten pâté recently than those affected by other strains. Government health warnings and suspensions of sale of pâté from the manufacturer involved were followed by a rapid decrease in the overall reported number of listeriosis cases, especially those due to the two epidemic serotypes. This provided circumstantial evidence that the UK outbreak observed was due to contaminated pâté from a single manufacturer. Two other outbreaks in France and Australia have also been linked to the consumption of contaminated pâté (Table 44–8).

A very large outbreak of foodborne listeriosis, linked by a case-control study and characterization of food isolates to the consumption of pork tongue in jelly, occurred in France in 1992. A total of 279 people were involved, with 92 cases involving pregnant women or neonates and 22 pregnancies ending in spontaneous abortion.[205] Of the 73 infants born, 7 died (9.6% fatality rate). In the other 187 cases (5 infants and 182 adults), 61% had a known pre-existing condition. The investigation was complicated by the fact that cases were spread across 79 departments within metropolitan France. Unfortunately, because of rapid consumption of product, there was no remaining pork tongue in jelly found in any of the patients' refrigerators.[252] However, the epidemic strain was isolated from 220 food products including delicatessen products (135), cheeses (40), meat and meat products (31), milk (1), other foods (13), and environmental samples (40). Delicatessen items that were contaminated included products in jelly, various hams and pâtés, and rillettes and sausages. Further illustrating the complexity of this investigation, most of these latter products were sampled in at least 30 retail stores. It is also apparent from this outbreak that cross-contamination of deli-style meats at the retail level occurred frequently and was responsible for causing additional cases of listeriosis. Among patients who did not eat the implicated food (pork tongue in jelly brand A), listeriosis was associated with the consumption of foods handled in the same stores where pork tongue brand A was sold.[252]

Another small outbreak that occurred in France in 1995 (Table 44–8) highlights the danger of eating cheese made from raw milk, especially for high-risk groups. In this outbreak, out of 20 cases, 11 perinatal cases occurred, resulting in 4 premature births, 2 spontaneous abortions, and 2 stillbirths.[206]

It is not known how many cases of foodborne listeriosis occur in which only a mild upper GI-type illness is seen, but the numbers may be substantial. In spite of the fact that mild cases of listeriosis are difficult to detect, there have been a number of outbreaks where, in the majority of cases, only mild symptoms were observed. The foods involved have been as diverse as shrimp,[426] chocolate milk,[413] and rice salad.[439]

In a Philadelphia outbreak,[451] multiple serotypes (4b, 1/2a, 1/2b, 3b) and isoenzyme types (11) were involved, and *L. monocytogenes* was not isolated from any of the foods eaten by the patients. Because of all these factors, and the high rate of enteric symptoms experienced by the patients, it was hypothesized that an infecting organism (bacteria and/or virus) may have precipitated the multiplication of *Listeria* present in the gut.[451] The hypothesis that individuals colonized with *L. monocytogenes* may develop GI infection after infection with another pathogen gained some support when shortly after an episode of shigellosis due to contaminated water, a previously healthy 57-year-old farmer was severely infected by listeriosis.[311]

44.6.2 Sporadic Foodborne Cases

The foods associated with sporadic cases of listeriosis include meats and poultry products (turkey frankfurters, chicken, sausages), dairy products (cheese, ice cream, raw milk, and human breast milk), vegetable products (pickled olives, mushrooms, vegetable rennet, alfalfa tablets), and seafood (smoked cod roe, fish).[74,142,341]

Several of the cases provided some interesting background information. In a case involving chicken nuggets,[265] the son of the mother involved was also affected by a mild, brief GI illness. Culture of the son's stools yielded several serotypes of *L. monocytogenes*, one of which was the same as that isolated from the mother (1/2a).

In a sporadic illness involving whey cheese,[15] a follow-up study[344] suggested that many people in the UK were likely to have ingested this contaminated cheese. Cheese samples from the incriminated plant frequently were contaminated with high numbers of *L. monocytogenes* ($>10^5$ cfu g^{-1}); however, phage-typing data showed that only 3% of the reported listeriosis cases occurring in Britain in 1987–1988 belonged to this phage type. Thus, many people may have been exposed to doses of *L. monocytogenes* in excess of 10^5 cfu g^{-1} without becoming seriously ill.

A sporadic listeriosis case in which turkey frankfurters were implicated was the first well-documented case involving a meat product.[23] The patient heated the product in a microwave oven for 45 to 60 seconds on high power before eating, suggesting that this microwave treatment was not sufficient to inactivate any *Listeria* present. As a result of this case, 600,000 pounds of turkey hot dogs were recalled by the hot dog manufacturer.[9] A case involving human breast milk was interesting in that some of the incriminated milk had been given to three Doberman puppies, all of whom became sick with vomiting, diarrhea, and blood in the stools. *L. monocytogenes* was isolated from the stools of one of the two surviving dogs.[482]

A retrospective study conducted in 1986–1987 of 154 listeriosis patients in six regions in the US found that sporadic cases were significantly more likely than controls to have eaten either uncooked hot dogs or chicken meat that was still pink.[450] A two-part follow-up study to examine the role of foods in sporadic listeriosis was conducted in 1988–1990 using a multistate population of 18 million people.[402,448] A case-control study found that soft cheeses (ie, Mexican-style cheese or feta) and foods from store delicatessen counters were significantly associated with an increased risk of listeriosis.[448] Undercooked chicken was also found to be a risk factor, but only in the most immunocompromised (ie, those affected by cancer, AIDS, organ transplants, immunosuppressive therapy) patients. This is in line with current theories that the MID for high-risk individuals is lower than that for the normal population. The second part of the study identified specific factors that would be likely to lead to an episode of sporadic listeriosis.[402] These included foods that contain high levels of *L. monocytogenes*, foods containing serotype 4b, and RTE foods. In this study, *L. monocytogenes* was found in the contents of two-thirds of the 123 refrigerators and in 11% of the total foods examined. The authors suggested that the majority of human listeriosis cases are foodborne.

There have been other outbreaks of listeriosis in which no foods have been implicated.[142] One of these outbreaks, which involved nine ill infants (4 to 8 days old) born after the delivery of an infant with early onset listeriosis, occurred in Costa Rica in 1989.[447] The newborn infants were bathed liberally over the entire body surface with mineral oil, which would come in contact with the baby's mouth, nose, and mucous membranes. The mineral oil, which was kept open on a cart, may have become contaminated through the splashing of contaminated amniotic fluid from the index case or via contaminated hands. Subsequent outbreak-associated cases were bathed with oil from the same container. Although many infants were exposed to the oil, invasive disease may have developed only in those babies who had aspirated the oil, with general anesthesia possibly increasing the risk of aspiration.[447] This hypothesis was partially supported by the finding of lipid-laden macrophages consistent with oil aspiration and traces of *L. monocytogenes* DNA in lung tissue from the one infant who died. As a result of the outbreak, the use of mineral oil for bathing neonates in Costa Rica was discontinued.

44.7 FACTORS AFFECTING GROWTH AND SURVIVAL, PARTICULARLY IN FOODS

44.7.1 Growth in Foods

L. monocytogenes can survive the manufacturing and ripening of many different types of cheeses[142] but tends to survive and grow best in soft and semi-soft cheeses, where levels as high as 10^5 and 10^7 cfu g^{-1} have been reported.[308,504] With soft cheese, the contamination is mainly localized on the rind surface.[17,435] This phenomenon is a result of the increased pH and proteolysis at the cheese surface due to the mold ripening process. During cheese manufacture, a highly significant correlation ($P < 0.005$) exists between *Listeria* growth, pH values > 5.5, and the absence of starter cultures.[188] Of 24 different types of cheese examined, ricotta and cottage cheese were the best and worst substrates for growth, respectively. Although *L. monocytogenes* was able to grow in 36.7% of the cheeses examined, there were a number of cheeses that caused gradual death of the organism at all temperatures (4° C, 8° C, and 30° C). Even though there was temperature-dependent growth of *L. monocytogenes* on a number of soft cheeses such as Brie and Camembert, little if any growth was observed in blue and white Stilton, Mycella, Chaume, and full-fat soft cheese with garlic and herbs stored at 3° C to 10° C.[17] Of 358 samples of various cheeses tested over a 2-year period, Mozzarella was the most contaminated (16%), followed by ricotta (9.1%) and gorgonzola (9%).[414] The incidence of *L. monocytogenes* in soft and semi-soft cheeses ranges from 0.5% to 15%.[142,308] A recent study of imported soft and semi-soft cheeses found that cheeses made from raw milk were much more frequently contaminated (42%) than cheeses made from heat-treated milk (2%).[308] In contrast, other studies have shown virtually no difference in the incidence of *L. monocytogenes* in cheeses made from raw or pasteurized milk.[52] The incidence of the organism in the final product will depend greatly on the amount of handling the cheese receives during manufacture. As compared with other bacteria, *L. monocytogenes* is a hardy microorganism that can survive for long periods in hard or semi-hard cheeses, or cheeses containing high levels of salt. As examples, *L. monocytogenes* was the only one of nine species of bacteria to survive the manufacture and ripening of Swiss semi-hard cheese[16] and survived much better in reduced-fat Cheddar-like cheese than did two species in the genus *Salmonella*.[348] The organism grew in white brined cheeses containing 10% and 16% NaCl and in white pickled cheese containing 8% NaCl.[1,271] Both Canadian and US requirements specify that hard cheeses made from raw milk should be maintained for at least 60 days at a temperature not less than 2° C. However, it is known that the organism can survive in some hard cheeses well past the 60-day hold period.[16,135,442]

Growth of *L. monocytogenes* on meats is highly dependent on the temperature of the meat, pH, type of tissue, and the type and amount of background microflora present. Glass and Doyle[198] found that the organism grew well on those meat products with a pH value near or above 6.0, while on meats near or below pH 5.0, the organism grew poorly or not at all. Poultry supported the growth of *L. monocytogenes* better than other meats, and roast beef, summer sausage, and hot dogs supported it the least. For roast beef, summer sausage, and hot dogs, the inhibitory factors appeared to be pH, combined pH and a_w, and liquid smoke, respectively.[198] *L. monocytogenes* also grew better at 0° C on vacuum-packaged beef of pH 6.0 than on meat of pH 5.6.[207] Regardless of storage temperature or pH, *L. monocytogenes* grew to higher levels on fat than on lean meat, probably as a result of a much shorter lag phase.[207] In contrast, other workers have found similar growth patterns of *L. monocytogenes* on both lean and fat beef tissue, with slightly longer lag periods occurring with the fatty tissue.[104]

L. monocytogenes can also grow in sausages,[417] on various types of wieners,[338] on VP and aerobic cooked beef,[240a] and on VP processed meats (corned beef, ham, and salami). High residual nitrite or lowered a_w reduced growth of *L. monocytogenes* on VP sliced corned beef and ham, particularly when stored at 0° C to 5° C;[208] *L. monocytogenes* grew faster on corned beef at pH $\approx$ 6.2, an a_w of 0.97, and with <5ppm NO_3 than on ham with a pH value of 6.6, an a_w of 0.97, and with 170 ppm NO_3. At 15° C, growth rates of *L. monocytogenes* and the background microflora, mainly lactic acid bacteria, were similar; at 5° C, *L. monocytogenes* grew at only one-third the rate of the lactic acid bacteria. The organism is also capable of growing on VP fresh ground beef at 4° C; however, the growth was slow, being more rapid at pH >6.0 and dependent on strain.[22] *L. monocytogenes* also was capable of growing well on liver pâté stored at 4° C and 10° C, and temperature was the only factor of several examined that exerted an effect on the growth rate.[154]

Conflicting results have been obtained on the effects of vacuum packaging of meats on the growth of *L. monocytogenes*. The organism grew equally well on VP and air-stored beef[104] but grew at a slower rate on VP chicken breasts than on film-wrapped samples.[72] *L. monocytogenes* was capable of growth on VP meat stored at 0° C, 2° C, 5° C, and 10° C; however, on high-pH beef packaged under 100% CO_2, it did not grow below 5° C.[193] The growth and survival of *L. monocytogenes* on meat stored under different gas atmospheres has recently been reviewed.[182] There have been conflicting reports on the ability of the organism to grow at high CO_2 levels, mainly because of the influence of factors such as pH, temperature, and microbial competition. Growth of *L. monocytogenes* at 4° C to 5° C has been observed on lamb and turkey roll at CO_2 levels of 50%,[144,382] whereas the organism was unable to grow at 6° C on chicken packaged under 30% CO_2 or at 4° C in an atmosphere of 75% CO_2.[219,526] However, adding 5% oxygen to the latter atmosphere resulted in growth of the organism. One can conclude that in most cases, *L. monocytogenes* will be able to grow in levels of CO_2 up to 50%. Growth of the organism in concentrations higher than this will depend mainly on the interplay between the gas atmosphere, pH, temperature, and microbial competition.

Most investigators studying the fate of *L. monocytogenes* in fermented sausages have found at least a 100-fold reduction in the organism during manufacture.[30,199,499] An exception was beaker sausage prepared without starter culture and held at 32.2° C for 16 hours, where the organism increased in numbers approximately 100-fold.[199] In contrast, no growth and a slight decrease of *L. monocytogenes* occurred in summer sausage made without starter culture.[30] These different observations most probably result from physical and chemical differences between sausages (ie, pH, a_w, salt and nitrate levels, background flora). In general, the organism appears capable of survival on meat regardless of treatment; freezing, surface dehydration, and simulated spray chilling do not appear to affect the survival of the organism on meat adversely.[104,261,272]

The organism is able to grow on a wide variety of seafoods such as smoked salmon,[216,240,432] crawfish,[120] cooked blue crab,[421] cold-smoked cod fillets,[108] cooked shrimp and lobster meat,[137] and smoked trout.[254] In the latter study, $>10^7$ *L. monocytogenes* g^{-1} were found on hot-smoked fish that had been inoculated with 3.1 to 4.5×10^1 MPN g^{-1} after smoking, and then stored at 8° C to 10° C for up to 20 days. No growth of the organism was seen, however, in products stored at 4° C. This re-emphasizes two important points for the control of *L. monocytogenes* in all RTE foods, ie, the prevention of postprocessing contamination and the storage of products at as low a temperature as possible throughout the food chain. In challenge studies with the organism on seafood or on other foods, the initial inoculum needs to be considered. Very low levels (≤ 10 cfu g^{-1}) of *L. monocytogenes* may fail to initiate growth at low temperatures.[108,137,398] Since most fish products naturally contain low levels of the organism, the organism would not be expected to grow to high levels on seafoods unless the product is temperature-abused. This may explain the small number of listeriosis cases linked to seafoods (Table 44–8).

There have been relatively few studies examining the growth of *L. monocytogenes* on whole or fresh-cut vegetables. *L. monocytogenes* was able to grow on shredded lettuce stored at 5° C, 12° C, and 25° C, although the increase was only about 1 log after 14 days at 5° C or 25° C, and about 3 logs after 14 days at 12° C.[477] In several trials, the organism did not grow or had died off after 14 days. The variation in results could not be fully explained, but was partially attributable to differences of pH and surface moisture, or competition from the bacterial flora. Carlin and Nguyen-the[70] found that, except for lamb's lettuce, *L. monocytogenes* grew on a variety of inoculated green leafy vegetables. No explanations could be found for the lack of growth on lamb's lettuce. Various salads left at 4° C for 4 days supported growth of *L. monocytogenes* (roughly 2-fold increase), indicating that the organism can survive and multiply during storage of refrigerated preprepared salads.[464] After an initial decrease in numbers, *L. innocua* grew on freshly shredded white cabbage treated with citric acid and sodium erythrobate and stored at 11° C for 21 days.[389] The increase in numbers of this organism was less for cabbage packaged in film with the highest oxygen transmission rate, implying that in some cases the aerobic micoflora can compete successfully with *Listeria* spp. Although *L. monocytogenes* was unable to grow on air-stored chicory endive stored at 6.5° C, it did grow on VP product stored at the same temperature;[14] mung-bean sprouts did not support the growth of the organism under either atmosphere. On minimally processed fresh endive, *L. monocytogenes* grew at all temperatures tested, with the greatest increase in numbers occurring on leaves that had undergone extensive spoilage and contained the greatest numbers of aerobic bacteria.[71] Growth rates of the organism (Table 44–10) were similar to those of the aerobic spoilage flora at 10° C and 20° C, but slower at 3° C and 6° C.

L. monocytogenes grew well at 15° C on fresh vegetables stored in air or controlled atmosphere (CA) (O_2:H_2:CO_2, 11% to 18%:79%:3% to 10%), increasing by about 4 logs within 6 days on asparagus, broccoli, and cauliflower.[29] At 4° C, however, it increased by only about 1 log on asparagus after 14 days, and decreased by about 0.5 logs after 14 days on broccoli and cauliflower. The observation that CA storage did not influence the rate of growth of *L. monocytogenes* is significant, since the storage life of the vegetables was considerably increased by the CA treatment. For vegetables on which the organism is capable of growing at 4° C (eg, asparagus), the increased shelf-life of the products allows extra time for *L. monocytogenes* to grow to significantly higher levels.

L. monocytogenes does not appear to be able to grow well on carrots unless the carrots are cooked.[33] Indeed, an anti-*Listeria* effect has been observed, with raw carrots stored at 5° C or 15° C spoiling before *L. monocytogenes* could grow. Broth-culture medium containing as little as 1% raw carrot juice substantially inhibited the growth of the organism, compared with the control. Oxygen appears necessary for the antilisterial activity of carrot, and thus reactive oxygen species may be involved. However, many free radical scavengers tested did not reduce the antilisterial effect.[370] The antilisterial effect of carrot juice was greatest in the pH range 5.0 to 6.4 and at low temperatures; NaCl levels up to 5% were protective.[36] In an examination of the possible inhibitory effects of carrot juice on *L. monocytogenes* on foods, treatment of lettuce with carrot juice levels of 20% or 50% caused significant reductions of the organism during storage at 5° C, retarded growth at 12° C, and was without effect at 20° C.[34] Carrot juice had little or no inhibitory effect on the growth of *L. monocytogenes* on Brie cheese or frankfurter homogenates.

44.7.2 The Effect of pH, Water Activity, and Temperature

The limiting parameters for growth of *L. monocytogenes* with respect to pH, a_w, temperature, and gas atmosphere can be seen in Table 44–9. The temperature range for growth is between –0.4° C and 50° C, with the optimum temperature being 25° C to 30° C.[263,515] The organism grows slowly on foods at refrigeration temperatures, with generation times ranging from 22 to 61 hours (Table 44–10); however, on foods that are temperature abused at 10° C, generation times of 10 to 13 hours usually are observed. Various predictive models are currently available that describe the growth of the organism under a wide range of different conditions.[524]

The rate of inactivation of the organism is dependent on pH, the acidulant, and its concentration. There have been a number of studies assessing the relative antimicrobial effects of organic acids such as citric, lactic, and acetic acids.[58,59,248,533] Recognizing the difficulty in comparing results because of differences in methodology, extrinsic parameters, and bacterial strains, discrepancies exist regarding the relative antimicrobial activity of various acids. For example, based either on an equivalent pH or an equimolar basis, the relative activity has been reported as acetic > lactic > citric.[7,248,533] Other investigators, however, have found the relative order to be citric ≥ lactic ≥ acetic acid, when expressed on the basis of molar concentration of undissociated acid.[58,533] To complicate matters further, organic acids such as citric acid can actually be protective at low (0.1 to 0.5%) levels.[58]

44.7.3 The Effect of Inhibitors

Antibacterial compounds that are either present naturally in food or that can be added to it[138] and inhibit *L. monocytogenes* in broth include monolaurin,[385] high molecular weight sodium polyphosphates,[534] and lysozyme either alone[260] or in combination with EDTA or apo-lactoferrin.[397] Compounds found to be effective in foods include sodium diacetate in turkey slurries;[445] sodium lactate in cooked beef,[358] liver, and bologna-type sausages;[417,458] trisodium phosphate on sliced beef tissue;[105] and monolaurin and lactic acid in crawfish tail meat homogenate.[386] The incorporation of organic acids in alginate gels to enhance antimicrobial effects increased the inhibition of *L. monocytogenes* resident on beef tissue.[461,463]

The effect of antibacterial compounds in foods, however, depends on the composition of the food and on temperature, pH, a_w, and interaction with other hurdles. For example, minerals or mineral-as-

Table 44–9 Limiting Parameters for Growth of *Listeria monocytogenes*

Parameter	*Minimum Conditions Allowing Growth*	*Reference*
Temperature	0.1 to –0.4° C	515, 516
	1.1° C	263
pH[a]	5.0 to 5.7; 4° C 4.3 to 5.2; 30° C	149a
	4.4 to 5.0; 10° C 4.4 to 5.2; 25° C	473a
	5.23; 4° C 4.62; 7° C 4.62; 10° C 4.59; 20° C 4.39; 30° C	189a
Water activity	4° C, 10° C; 0.93—NaCl 15° C; 0.91—glycerol, NaCl 30° C; 0.90—glycerol 40° C; 0.93—glycerol	151a
	4° C 0.92—glycerol 0.93-0.96—sucrose 0.94—NaCl	485a
	30° C 0.90—glycerol 0.92 to 0.96—sucrose 0.92—NaCl	485a
	21° C 0.924—NaCl 0.925—sucrose 0.911—glycerol	377a
Gas atmospheres	7° C, pH 5.5, 50% CO_2[b]	154a

[a] Values vary depending on acidulant used.

[b] Growth not observed in BHI broth at 4° C under same conditions.

sociated components (eg, Ca^{2+}, Mg^{2+}) protected *L. monocytogenes* from inactivation by lysozyme and heat in milk.[276] No simple relationship was found between the listericidal nature of various marinades and the presence or absence of green shell mussels.[55] The antibacterial action of mint essential oil against *L. monocytogenes* in various model foods was dependent on the oil concentration, food type, pH, food composition, and storage temperature.[488]

Several general reviews have been published on the activity of starter culture bacteria and/or their bacteriocins against *L. monocytogenes*,[235,249,365] and more specific ones on mode of action,[2] potential use in meat[346] and dairy products,[196] bacteriocins produced by *Leuconostoc*,[224] and regulatory considerations.[160] Specific examples of the use of lactic acid bacteria or their bacteriocins are shown in Table 44–11. Some of the main issues are discussed in Chapter 12.

44.7.4 The Effect of Heat Treatment on Survival

The outbreak of listeriosis in Massachusetts in 1983, which was associated with the consumption of pasteurized milk, raised the question of the heat resistance of *L. monocytogenes*. In studies with *L. monocytogenes* (Scott A) suspended in raw milk and heated in sealed glass tubes, *D*-values of 19.9, 3.0, and 0.9 seconds at 53.3° C, 68.9° C, and 71.7° C, respectively, were obtained, giving a *z* value of 6.3° C.[50] It was concluded that the organism would not survive 71.7° C for 15 seconds, which are the conditions for high-temperature, short-time (HTST) pasteurization. Doyle et al.[121] infected four cows with *L. monocytogenes* (Scott A) and performed pasteurization trials on the milk obtained from these cows, which contained the organism within polymorphonuclear leukocytes. *L. monocytogenes* cells were recovered from six of nine trials in which the milk was heated from 71.7° C to 73.9° C, but were not recovered from milk that was heated from 76.4° C to 77.8° C. Initial levels of the organism in sonicated or homogenized milk ranged from 1.0×10^2 to 2.6×10^4 cfu ml^{-1}. They suggested that the discrepancy between this result, and that of Bradshaw et al.[50] was mainly due to the intracellular location of the bacteria. An extensive study involving sterile whole milk, in which *L. monocytogenes* cells were either freely suspended or located within bovine milk phagocytes, showed that the intracellular position of the organism did not significantly increase thermal resistance.[64] However, they did demonstrate that the HTST conditions did not provide an ad-

Table 44–10 Generation Times of *Listeria monocytogenes* in Foods[71,241,471]

Food	*Temperature (°C)*	*Generation Time (h)*	*Reference*
Milk	4	25	150
	9.5	11	57
	10	10.8	150
Various dairy products	4	30 to 46	434
	21	1.8 to 2	434
Ice cream	4	21.6	437
	9.5	11	57
	21	1.1	437
Chicken broth	2.5	35	516
	9.3	6	516
Lettuce juice	4.5	25	357
	10	11	357
Broad-leaved endive	3	35, 57	71
	6	19, 31	71
	10	7.6, 12	71
	20	4.4, 3.9	71
Crawfish tail meat	0	72.2	120
	6	17.0	120
	12	6.9	120
Whey	6	14.8 to 21.1	436
Roast beef	–1.5	129.0	241
	3	37.6	241
Corned beef	0	110.0	208
Raw cabbage	5	43.0	35
Cooked meat	5	44 to 61	471
Ham	5	33.2	208
	10	13.4	208
	15	6.1	208
Asparagus	15	9.7	29
Broccoli	15	7.2	29
Cauliflower	15	7.2	29

Table 44–11 Some Examples of Starter Cultures or Bacteriocins with Anti-*Listeria* Activity

Organism	*Bacteriocin*	*Source of Isolate or Food System Examined*	*Reference*
I. BROTH OR AGAR SYSTEMS			
Lactobacillus curvatus	Curvaticin FS47	Ground beef isolate	184
Carnobacterium divergens	Divercin V41	Fish isolate	401
Carnobacterium piscicola	Piscicocin V1	Fish isolate	401
Brevibacterium linens	Linenscin OC2	Orange-cheese isolate	326
Streptococcus thermophilus	Thermophilin	Yogurt isolate	510
Lactobacillus plantarum	Plantaricin C	Cheese isolate	203
Brochothrix campestris	Brochocin-C	ATCC*	462
Leuconostoc carnosum	Carnosin	Sausage isolate	502
Lactobacillus sake	Sakacin A	NK[†]	232
Leuconostoc mesenteroides subsp. *mesenteroides*	Mesenterocin 52	Raw milk isolate	333
II. IN FOOD			
Carnobacterium piscicola	Carnocin CP5	Skimmed milk	334
Lactobacillus sake	Sakacin K	Fermented sausages	242
Pediococcus acidilactici	Pediocin	Frankfurters	31
		Sausages	166
Lactobacillus bavaricus	Bavaricin Mn	Beef	527
Leuconostoc spp.	NK	Shrimp	259
Lactobacillus plantarum	NK	Shrimp	259
III. DIRECT ADDITION OF BACTERIOCINS			
N/A	Pediocin 5	Milk	237
N/A	Nisin	Cottage cheese	159
N/A	Nisin	Ice cream	99
N/A	Nisin	Raw pork	134
N/A	Nisin	Poultry	325
N/A	Enterocin 1146	Milk	391
Pediococcus acidilactici M	Crude dried bacterium powder	Kimchi	79

* ATCC, American Type Culture Collection.
[†] NK, not known.

equate *D* process, as the predicted $D_{71.7° C}$ value for intracellular slug-flow heat exchange studies was 4.1 seconds. This suggested a possibly unsafe 3.7 *D* kill for a minimum (71.7° C; 15 seconds) commercial pasteurization, whether the organism was located extra- or intracellularly. In a later report, Garayzabal et al.[181] showed that although *L. monocytogenes* was not isolated from inoculated raw milk that had been treated in a pilot plant-size pasteurizer at temperatures ranging from 69° C to 73° C for 15 seconds if the isolation was attempted immediately after the thermal treatment, it could be isolated from those samples heated from 69° C to 73° C after incubation for from 2 days to 3 weeks. This emphasizes the importance of the isolation techniques used for studying heat-stressed organisms. In a study again involving both extra- and intracellularly located *L. monocytogenes* in naturally contaminated milk, Farber et al.[147] reported that though the organism was recovered from milk heated to the 60° C to 66° C temperature range, no viable listeriae were recovered if the isolation was done immediately after treatment at 69° C and above for 16.2 seconds.

The heat resistance of *L. monocytogenes* in dairy products has been reviewed.[117,136,214,320] Mackey and Bratchell[320] summarized the data and found that according to their models, HTST and vat pasteurization of milk would achieve a 5.2 and 39 *D* reduction in the numbers of *L. monocytogenes*, respectively. The *z*-values calculated from heating in sealed tubes and in a slug-flow heat exchanger were 6.1° C and 7.4° C, respectively. These values were close to the overall (all foods and broths) calculated *z* values of 6.7° C to 6.9° C[320] and are close to those values found for *L. monocytogenes* in meats and seafood (Table 44–12).

Two factors known to play a major role in the recovery of thermally treated listeriae led investigators to reappraise the data on the effect of pasteurization on the survival of the organism. The first is called the heat-shock response. If *L. monocytogenes* is exposed to sublethal temperatures of 44° C to 48° C for 20 to 60 minutes before being subjected to the final test temperature, the cells acquire an enhanced thermotolerance. This has been demonstrated both in a milk[156] and in a meat system.[141] In the meat system, heat-shocked cells cooled to 4° C maintained their thermotolerance for at least 24 hours after heat-shock.[141] Cells grown at high temperatures appear to become more heat-resistant. For example, *L. monocytogenes* cells grown at 43° C were more thermotolerant than cells grown at lower temperatures or cells that had been heat-shocked at 43° C for 5, 30, or 60 minutes.[282] In a study of the effect of growth temperature on the thermal resistance of *L. monocytogenes* in HTST pasteurized whole milk following inoculations ranging from 3.0×10^4 to 1.2×10^5 cfu ml^{-1}, the organism survived the pasteurization process in 0 of 4, 2 of 5, and 4 of 4 trials when grown at 30° C, 39° C, and 43° C, respectively.[152] The numbers of *L. monocytogenes* surviving were 0.36 to 0.92 MPN ml^{-1} for cells grown at 39° C, and ranged from 0.03 MPN ml^{-1} to 1.3×10^2 cfu ml^{-1} for cells grown at 43° C. The authors speculated that two different fac-

Table 44–12 Heat Resistance of *Listeria monocytogenes* in Meat, Chicken, and Seafood

Product	*Temperature (° C)*	*D value (min)*	*z value (° C)*	*Reference*
Beef	60	3.8	7.2	321
	70	0.14		
Chicken leg	60	5.6	6.7	321
	70	0.11		
Chicken breast	60	8.7	6.3	321
	70	0.13		
Ground beef	60	3.12	5.3	149
Fermented sausage mix	60	16.7	4.6	
Meat slurry	60	2.54		48
	70	0.23		
Chicken	60	5.29[a], 5.02[b]	6.7,[a] 7.4[b]	185
	70	0.16,0.20		
Beef steak	60	8.32,6.27	5.98,5.98	185
	70	0.20,0.14		
Liver sausage slurry	60	2.42	6.2	37
Chicken gravy	60	3.13[c]	5.18	236
		7.07[d]	6.10	
Meats (predicted value)	60	3.82	6.8	321
	70	0.13		
Raw liquid whole egg	60	1.46[d]	6.6[d]	163
Crabmeat	60	2.61	8.4	218
Mussels	60	5.49	4.3	55
Crawfish tail meat	60	1.98	5.5	120
Salmon fillets (vacuum packed)	60	4.23	6.7	27
		4.48	5.6	
Cod fillets (vacuum packed)	60	1.95	6.1	27
		1.98	5.7	
Lobster meat	60	2.39	5.0	62

[a] Strain Scott A
[b] Strain 11994
[c] Strain V7
[d] Average of five strains

tors, heat-shock proteins and a highly viscous bacterial membrane, may act individually or interactively at high growth temperatures to confer an enhanced thermotolerance on the cells. In addition to heat, other stress factors such as starvation, ethanol, acid, and H_2O_2 can also lead to enhanced thermotolerance of *L. monocytogenes*.[312] A comparison of $D_{56° C}$ values showed that, compared with control cells (D value = 1.0 minute), the stress of starvation resulted in the highest D value (13.6 minutes). Cells held at lower temperatures (4° C > 10° C > 30° C) maintain their thermotolerance for a longer time than those held at higher temperatures.[262] For example, cells both grown and held at 4° C for 48 hours after a heat-shock of 30 minutes at 46° C were still 2-fold more heat-resistant than nonheat-shocked cells grown at 4° C. Increased thermotolerance of *L. monocytogenes* was observed during slow rates of heating, which was analogous to the induction of the heat-shock response.[478] Maximum thermotolerance was observed at rates of heating of ≤ 0.7° C/minute, while at rates ≥ 5° C/minute enhanced heat tolerance was not observed.

The second factor influencing recovery concerns the methodology used for recovering heat-stressed organisms. Crawford et al.[89] studied the recovery of *L. monocytogenes* cells from inoculated milk heated at 71.7° C in a slug-flow heat exchanger, comparing the effects of enrichment in a nonselective broth (NSB) to the Food and Drug Administration (FDA) and US Department of Agriculture (USDA) enrichment methods. The D values obtained were 2.7 ± 0.8, 1.3 ± 0.4, and 0.7 ± 0.2s for the NSB, FDA, and USDA enrichments, respectively. The use of strict anaerobic techniques can lead to significantly greater recovery of heat-stressed cells than enumeration in the presence of oxygen.[282] For example, $D_{62.8°}$ C values for cells grown at 43° C and recovered anaerobically were at least 6-fold greater than those for cells grown at 37° C and enumerated aerobically. The oxygen sensitivity of heat-stressed *L. monocytogenes* cells was believed to be due to the inactivation of the enzymes catalase and superoxide dismutase during heating.[282] A novel anaerobic method for the recovery of heat-injured *L. monocytogenes* has been developed that uses cysteine and N_2 purging to reduce or eliminate oxidative stresses during enrichment.[281,349] In summary, a reduction in numbers of *L. monocytogenes* in milk of at least 3 logs can be expected after a minimum HTST pasteurization.

In the US, pasteurization standards for frozen dessert mixes require heating for either 30 minutes at 68.3° C or 25 seconds at 79.4° C. $D_{60° C}$ values obtained for nine ice cream mixes ranged from 2.13 to 4.79 minutes, leading to the conclusion that these pasteurization guidelines appear adequate to ensure the inactivation of *L. monocytogenes* in ice cream.[233] The use of common ice cream stabilizers such as guar gum and carrageenan resulted in an increased thermal tolerance of the organism in ice cream mixes.

Information on the heat resistance of *L. monocytogenes* in meats and seafood is shown in Table 44–12. In general, the addition of meat-curing ingredients provides some protection of the organism,[141,531] although this protection may not be evident at temperatures of ≥ 67° C.[532] Chicken gravy was inoculated with *L. monocytogenes* at a level of 10^5 cfu ml^{-1}, and then heated to 50° C, 55° C, 60° C, or 65° C, either immediately after inoculation or after 1, 3, 5, or 10 days of storage at 7° C. $D_{50° C}$ values for strains Scott A and V7 were 119 and 195 minutes at day 0 and 115 and 119 minutes at day 10, respectively. $D_{65° C}$ values were 0.48 and 0.19 minutes at day 0 and 0.016 and 0.007 minutes at day 10, respectively.[236] In contrast, the heating process used commercially for cured and uncured turkey rolls (internal temperatures of 68° C and 74° C, respectively) and battered chicken nuggets (internal temperature of 71° C) was shown to be adequate to reduce 10^7 cfu ml^{-1} of *L. monocytogenes* to below the detection limit.[303] The heating of hot dogs to an internal temperature of 160° F (71° C) resulted in an approximate "3D" reduction in numbers of *L. monocytogenes*.[535] If these products were heated slowly to the final temperature at less than 5° C per minute, the above conclusions would need to be modified to take account of the effect of heat-shock.[14,415,478] Mackey et al.[321] formulated an equation for calculating processing times based on a "7D" inactivation of *L. monocytogenes* in meat, which takes into account the pronounced shoulders on their survivor curves. The equation, $\log_{10}$ processing time = 10.3943 – 0.14618t where t = heating temperature, predicted, for example, that 70° C for 1.45 minutes would be equivalent to a "7D" kill. The authors agreed with Gaze et al.[185] that for the majority of cases, heating at 70° C for 2 minutes would be sufficient to inactivate any *L. monocytogenes* present in raw meat.

In liquid whole egg, the minimal pasteurization schedule recommended by the USDA for this product (60° C; 3.5 minutes) would result in between 2 and 4 log reductions in numbers of *L. monocytogenes*;[24,163,164] however, in a similar product, supplementation with 10% NaCl was protective, resulting in only a 0.2 to 0.6 log reduction after heating.[24] It can be inferred that the incorporation of salt or sugar into egg products will protect bacteria against heat, and the minimum pasteurization schedules for products containing these substances are greater than for those for eggs without these additives. Frying eggs "sunny side up" only slightly decreased (0.4 logs) numbers of *L. monocytogenes*, but scrambling eggs was much more effective, in one instance reducing numbers of the organism by 3 logs.[49]

In a comparison of the heat-resistance in physiological saline of different species of *Listeria* and different serotypes and strains of *L. monocytogenes*, $D_{60°\,C}$ values for *L. monocytogenes* ranged from 0.72 minutes (serovar 1/2a) to 3.1 minutes (serovar 3b).[473] A study of the heat resistance of 27 strains of the organism in tryptone soya broth gave $D_{57°\,C}$ values ranging from 6.5 to 26 minutes.[321] Serotype 1 strains may be more heat-resistant than those of serotype 4.[300] Although far from conclusive, there is some evidence that *L. innocua* may be more heat-resistant than *L. monocytogenes*.[165] For those laboratories not willing or not equipped to work with foodborne pathogens, the latter authors have developed an antibiotic-resistant (chloramphenicol and erythromycin) strain of *L. innocua* for both challenge and heat-resistance studies.

44.7.5 The Effect of Irradiation on Survival

Several studies have examined the irradiation resistance of *L. monocytogenes* in foods and in broth[418] and indicate that for a vegetative cell, the organism is quite radiation-resistant. Of the seven pathogenic bacteria examined, *L. monocytogenes* was the most radiation-resistant in saline solution and chicken breast meat, with D_{10} values of 0.16 to 0.38 kGy.[110] A dose of 3 kGy was necessary for the inactivation of 10^3 cfu g^{-1} of *L. monocytogenes* in air-packed frozen chicken meat.[266] In ground beef, D_{10} values ranged from 0.507 to 0.610 kGy.[361] It was calculated that a dose of 2.5 kGy would inactivate 4.1 logs of *L. monocytogenes* and 5.12 logs of *S. aureus* per gram of ground beef. A treatment dose of 2.6 kGy inactivated 4 logs of the organism in Camembert cheese, with D_{10} values of 0.50 ± 0.05 kGy reported.[47] No statistically significant differences were observed for the radiation resistance of *L. monocytogenes* in various meats; D_{10} values at 5° C of 0.45, 0.47, 0.48, 0.50, and 0.47 kGy were obtained for beef, lamb, pork, turkey breast, and turkey leg, respectively.[491] *Salmonella* were more radiation-resistant than listeriae in these products, with D_{10} values ranging from 0.51 to 0.71 kGy. In a shrimp suspension and with treatment temperatures of –20° C and 0° C, D_{10} values ranging from 0.67 to 0.84 and 0.28 to 0.36 kGy, respectively, were obtained depending on the strain and recovery media used.[51]

Despite the basic differences between electron beam treatment and irradiation by gamma rays from ^{60}Co, their lethal effects on microorganisms are thought to be similar. Electron beam doses of 1.1, 2.2, and 2.9 kGy gave reductions of 2.21, 2.77, and 4.65 logs g^{-1}, respectively, of *L. monocytogenes* in VP (sous-vide) chicken breast.[454] Heat treatment and irradiation were synergistic in their action against *L. monocytogenes*, and this treatment led to an extension of product shelf-life of at least 2 weeks. In a study of electron beam irradiation sensitivity of five strains of *Listeria* spp. inoculated into ground pork, *L. innocua* (D_{10} = 0.638 kGy) was significantly more radiation-resistant than three strains of *L. monocytogenes* (D_{10} = 0.424 to 0.447 kGy) or *Listeria ivanovii* (D_{10} = 0.378 kGy).[487] The authors considered that a total dose of 1.5 to 4.5 kGy, which is currently being considered by the FDA for the irradiation of beef and pork, would be adequate to eliminate *L. monocytogenes* from pork (ie, would be sufficient to give approximately a 3.4 to 10.1 log reduction in numbers).

44.7.6 The Effect of Non-Thermal Treatments on Survival

The desire of consumers for fresher RTE convenience foods that require less chemical preservatives and require minimal processing has led the investigation of processes such as high electric-field pulses, oscillating magnetic-field pulses, intense light pulses, microwaves, and high-pressure treatments. Several studies have examined the sensitivity of *L. monocytogenes* to high hydrostatic pressure treatment[69,292,395,480] and microwaves.[215,225]

44.7.7 Heat-Induced Injury

Many of the selective media currently in use for the isolation of *L. monocytogenes* may not be satisfactory for the recovery of injured cells; investigators have tried to improve the recovery of heat-injured cells by the addition of various compounds. Catalase,[392,393] Oxyrase®,[393] and cysteine[281,349] have been found by some investigators to maximize the recovery of heat-stressed cells in broth, but other workers have not seen any benefit from the addition of catalase.[322,467]

A repair enrichment broth for resuscitating heat-injured *L. monocytogenes* was developed,[65] which contains trypticase soy broth, glucose, yeast extract, magnesium sulfate, ferrous sulfate, sodium pyruvate, and buffers. Heat-injured *L. monocytogenes* repaired themselves in both whole and 2% milk, but the repair process was very sensitive to minor changes in temperature.[356] For example, at 4° C, 10° C, and 26° C, the repair process took 16 to 19 days, 4 days, and 12 hours, respectively. Mackey et al.[322] speculated that although repair may be more rapid at 37° C, it may not recover as many injured cells because they found that the optimal repair temperature was 20° C to 25° C. Obviously, more work is needed in the whole area of heat and/or stress-induced injury.

44.8 PRINCIPLES OF DETECTION AND ISOLATION

Many culture methods for isolation of *L. monocytogenes* from environments such as food have now been developed,[153,314,337] and alternative methods using monoclonal antibodies (Mabs),[91] DNA probes,[98] or PCR[43] are being reported.

44.8.1 Cultural Methods

In the USDA detection procedure for use with meat and poultry,[337] the primary enrichment broth was based on Donnelly and Baigent's[118] *Listeria* enrichment broth (LEB) but was modified to contain nalidixic acid at 20 mg l^{-1}. This was modified further to form the secondary enrichment broth containing acriflavine at 25 mg l^{-1}. The primary broth containing the sample was incubated for 24 hours at 30° C, after which a portion was transferred to secondary enrichment broth. An improved selective medium (LPM agar) was developed containing lithium chloride, phenylethanol, and moxalactam, a broad spectrum antibiotic inhibitory to many Gram-positive and Gram-negative bacteria, as selective agents. The USDA now uses Fraser broth (FB) as the secondary enrichment broth and modified Oxford agar (MOX)[337] as the selective medium. The use of FB facilitates *Listeria* identification as the presence of esculin, and ferric ammonium citrate causes a darkening of the broth in the presence of *Listeria* species. Because many false-negative results have been observed due to reliance on this darkening, the USDA method now calls for all FB cultures to be subcultured to MOX plates after 24 hours of incubation.[10]

Other useful selective agar media include RAPAMY, PALCAM (PAL),[503] and Oxford agar (OXA).[93] Although no one medium has clearly emerged as superior, PAL medium appears to be preferred in Europe, while LPM, OXA, and MOX media are the most widely used in North America. Cultural methods for *Listeria* detection have been reviewed by Curtis and Lee.[92]

The highly selective LPM agar formed the basis of the FDA *Listeria* detection method,[314] which is recommended for the detection of the organism in foods other than meats. Nalidixic acid and acriflavine are used in the FDA enrichment broth (EB), which, in addition, contains cycloheximide, an antifungal agent used to suppress the yeasts and molds often present in foods. Samples are inoculated into EB, and incubated at 30° C for 2 days. At 1 and 2 days, samples are plated onto a selective medium, which was originally modified McBride agar; however, since 1990, LPM and OXA agars have been used.[155] The plates are then incubated for 24 to 48 hours at 30° C and 35° C respectively, for detection and identification.

The Health Protection Branch (HPB), Canada, detection method was developed for all food and environmental samples.[153] Samples are inoculated into LEB and incubated for 48 hours at 30° C. At both 24 and 48 hours, modified Fraser broth (MFB) is inoculated from the LEB culture and incubated 24 hours at 35° C before plating onto OXA agar and one of either LPM, MOX, or PAL for detection and identification.[153]

An HPB enumeration method involves plating the sample on any two of LPM, OXA, MOX, or PAL agars, followed by incubation of the LPM plate at 30° C, and the other selective agar media at 35° C.[145] A presumptive identification and enumeration, based on the colony appearance, are followed by confirmation procedures.

44.8.2 Alternative and/or Rapid Methods

To monitor the incidence of *L. monocytogenes* in foods, methods based on immunological, genetic, or electrical techniques for rapid detection and identification of food isolates need to be designed.[142]

The production of Mabs specific for the genus *Listeria* was first reported in 1987.[139] Recently, the production of a Mab with a high degree of specificity for *L. monocytogenes* 4b was reported.[269] Because this serotype accounts for many of the large foodborne outbreaks of listeriosis, the Mab is of potential interest as the basis of an immunological method.

Methods based on enzyme-linked immunosorbent assay (ELISA) using an antibody (Ab)-antigen (Ag)-Ab sandwich technique are available commercially as Listeria-Tek™ ELISA (Organon Teknika Corp., Durham, NC)[91] and TECRA™ Listeria VIA (Bioenterprises Pty Ltd., Roseville, Australia) kits. In both cases, a sample of the incubated EB is placed in a precoated microwell and incubated for 0.5 to 1 hour, during which *Listeria* cells are captured by the

Ab. Enzyme-conjugated Ab is added at the same time (Listeria-Tek kit) or after a washing step (TECRA kit). After washing, a chromogenic enzyme substrate is added and the color intensity measured with a photometer, yielding a positive/negative result.[289,517] In one comparative study, the results using the TECRA kit agreed completely with the results by a cultural method, while the *Listeria*-Tek kit gave a 23% (7 of 30 samples) false-negative rate.[289] Because the detection limits are 10^4 to 10^5 cfu ml^{-1}, an enrichment step is required for these methods, which increases their overall detection time. An automated ELISA-based system is found in the VIDAS™ Listeria Test (bioMérieux, Marcy-l'Étoile, France), in which the instrument requires no further manipulations after the enriched sample is placed in the reagent strip (Technical Literature, VIDAS Listeria Test, bioMérieux). It uses a genus-specific Ab in a sandwich technique in which alkaline phosphatase is conjugated to the second Ab, catalyzing the breakdown of the fluorescent substrate. The results compared with a cultural method showed 100% sensitivity and 91% specificity (Technical Literature, VIDAS Listeria Test, bioMérieux).

An interesting adaptation of the ELISA detection method has been developed in the coating of magnetic beads with an anti-*Listeria* Ab, as in the DYNABEADS™ (Dynal Inc., Great Neck, NY) and Listertest™ (Vicam, Somerville, MA) kits. The Ab-coated magnetic beads are added to the sample and incubated with gentle mixing for 1 to 2 hours (Technical Literature, DYNABEADS, Dynal; Listertest, Vicam). The beads, with their bound *Listeria* cells, are placed in a magnetic field for separation from the sample and washing. Once separated, cultural or rapid detection methods can be applied, thus eliminating the need for an enrichment step.[250]

The detection of *L. monocytogenes* by colony hybridization with a radioactive-labeled DNA probe, which is based on the *hlyA* gene and is specific for *L. monocytogenes*, was first reported by Datta et al.[97] From sequencing information obtained from this probe, a 20-mer oligonucleotide probe was developed and used as a synthetic probe in a colony hybridization method to identify β-hemolytic *L. monocytogenes* in ground beef, with 100% specificity.[360] To avoid the problems associated with the use of radioactivity, the natural probe has since been chromogen-labeled with horseradish peroxidase and used in the enumeration of the organism in foods with 100% sensitivity.[98] The probe also has been labeled with alkaline phosphatase and used for direct colony hybridization on hydrophobic grid-membrane filters (HGMF).[530] In a comparison with the HPB standard method,[153] the HGMF DNA probe method was highly specific in discriminating *L. monocytogenes* from other *Listeria* species present in the food samples. The use of anti-DNA-RNA Mabs specific for hybrid nucleic acid (HNA) molecules formed a new approach to the detection step in a DNA probe method for *Listeria* species.[162] Using a double-sandwich ELISA to detect HNA formation, the authors were able to detect as little as 2.5 pg of target RNA, or 5×10^2 cfu ml^{-1} of *Listeria* species in foods.

Commercial hybridization assay kits are now available, based on DNA probes targeted to genus-specific regions of 16S rRNA (Gene-Trak Systems, Framingham MA) or *L. monocytogenes*-specific rRNA (Gen-Probe, San Diego, CA). The Gene-Trak™ probe solution, added after enrichment of the food sample, contains two probes that hybridize to adjacent regions of the target *Listeria* rRNA. The polyadenylate tail on the capture probe hybridizes to plastic dipsticks coated with complementary polydeoxythymidine. The detector probe also contains a ligand, fluorescein. When the dipstick is transferred to an enzyme-labeled antifluorescein Ab, the presence of the target-probe complex is visualized by means of a substrate.[90] When the performance of the Gene-Trak method was compared with the USDA or FDA cultural methods and used on the appropriate food, the agreement between methods was 82%.[90] Gene-Trak has since developed an *L. monocytogenes*-specific probe to use as the capture probe (Technical Literature, Gene-Trak).

Using PCR for the detection of *L. monocytogenes* in food and concentration of enrichment broths by centrifugation, the lower limit of detection in most foods was about 10 bacteria $(10g)^{-1}$; all the naturally contaminated foods in which *L. monocytogenes* was detected by cultural testing were also positive by the PCR test.[372] The total analysis time for the PCR test was 32 hours. A method for direct extraction of DNA from foods and testing by PCR was described that enabled the detection of 10^3 to 10^4 cfu *L. monocytogenes* g^{-1} of food.[103] The primers that have been used for the detection of *L. monocytogenes* are shown in Table 44–13. *L. monocytogenes* was among the pathogenic organisms detected in foods after the direct application of PCR to extracted DNA, the bacterial cells being detected at 10^3 to 10^4 cfu ml^{-1}.[103] An alternative to analysis by gel electrophoresis is to use an RNA probe that hybridizes specifically to the desired PCR product and is detected by an anti-RNA-DNA hybrid Ab.[42] A specific oligonucleotide coupled to paramagnetic beads has been used to isolate listerial DNA from foods.[374] The isolated DNA was amplified by PCR, with a detection

Table 44–13 Genes Targeted in PCR for Detection of *Listeria monocytogenes*

Gene	Food/Pure Culture	Sensitivity (cfu g^{-1})
hly	poultry	1
	ground beef	1 to 10
	milk	1 to 10
	pasteurized milk	1
	soft cheese	1
	milk, dairy	1
iap	PC[a]	
prfA	PC	
plcB	PC	
lmaA	PC	
inlA	PC	
16S rRNA	poultry	10
	dairy[b]	NK[c]

[a] Pure culture.
[b] See [103].
[c] Not known.

Source: Data from A.R. Datta and M.A. Benjamin, Advances in Nucleic Acid-based Techniques for Detection of Listeria Monocytogenes, *Proceeding of the 12th International Symposium on Problems of Listeriosis*, October 2–6, 1995, Perth, Australia.

limit of 1 to 10 cfu 10 g^{-1} of food.[25] PCR can be affected by the presence of inhibitors in samples and the technique detects both live and dead cells. It seems that, at the present time, PCR is preferably used as a screening technique.

In a comparison between Gen-Probe DNA probe and a PCR technique, although both techniques showed high specificities, the sensitivity of the Gen-Probe method was low.[373] The report concluded that PCR applied after a two-step enrichment process was the most sensitive method for detecting *L. monocytogenes* in soft cheeses.

Electrical detection methods measure changes in conductance or impedance of the medium during microbial growth.[221] These methods detect *Listeria* species in hours rather than days. Media specific for the growth of this pathogen are required for the successful application of this method.[68]

44.8.3 Identification Methods

Miniaturized multitest assays are commercially available for the identification or confirmation of presumptive *Listeria* isolates to the species level. The API-Listeria™ test (API-bioMérieux Inc., Marcy-l'Étoile, France) uses 10 biochemical tests for identifying *Listeria* isolates to the species level, following a 24-hour incubation. When combined with the detection of β-hemolytic activity on blood agar plates, all of the 70 *Listeria* strains tested were correctly identified.[177] Tests of 65 strains of *Listeria* species showed that the API-ZYM system (API-bioMérieux), which uses detection of 19 constitutive enzymes, could be used for the rapid (4 hour) confirmation of *L. monocytogenes*.[100] In the Mast ID™ system (Mast Laboratories, Bootle, UK), carbohydrate substrates and a pH indicator are incorporated into agar plates, which can be used to screen up to 21 isolates per plate to identify *Listeria* species.[273] The MICRO-ID™ LISTERIA system (Organon-Teknika) uses 15 biochemical tests to identify *Listeria* to the species level after 24 hours incubation. In the case of 99% (409 of 414) of the strains, the cAMP test was also needed for correct identification of strains.[20] A collaborative study involving 13 laboratories, each testing 97 food and environmental isolates, evaluated the AutoMicrobic System (bioMérieux Vitek Inc). This kit, which uses up to 30 biochemical tests, is scanned hourly in the system for turbidity and/or color changes and correctly identified 91% of *Listeria* species and 100% of non-*Listeria* species.[217]

A colony-confirmation test, based on a DNA probe, forms the basis of the commercially available Accuprobe™ *L. monocytogenes* Culture Identification Reagent Kit (Gen-Probe). Using an acridinium ester-labeled DNA probe, this chemiluminescent method was reported to be 100% sensitive and 100% specific when tested on a total of 70 *Listeria* strains[375] for the rapid confirmation of 296 suspect *L. monocytogenes* colonies.[387]

PCR used in the identification of presumptive *L. monocytogenes* isolates during routine analysis by the HPB method showed 100% sensitivity and 100% specificity.[43]

44.8.4 Tests for Virulence

Since *L. monocytogenes* was first isolated, workers in the field have stated the need for simple, rapid procedures to differentiate isolates of this species from nonpathogenic *Listeria* spp., without resorting to animal inoculation. Several procedures that demonstrate hemolysin production were proposed to meet this need. The simplest test for the presence of β-hemolysis is the appearance of zones of clearing on horseblood agar plates. However, these are often difficult to interpret with *L. monocytogenes* and *L. seeligeri* because strains vary in the intensity of their reaction. Recently, hemolytic ceftazidime lithium chloride agar (HCLA) was developed as a differential medium for *L. monocytogenes*.[410] When horseblood agar is incorporated into the agar overlay, which is poured over the selective medium containing lithium chloride, colistin methane sulfonate, and ceftazidime, *L. monocytogenes* forms blue-gray colonies surrounded by zones of β-hemolysis. The cAMP phenomenon, originally noted with Group B streptococci, was adapted for use with *Listeria* species by the addition of a streak of *Rhodococcus equi*, which enabled *L. ivanovii* to be differentiated from *L. monocytogenes* and *L. seeligeri* by their opposite reactions with the hemolysins of the two test organisms.[142]

The chick embryo test, where the chorioallantoic membrane of 10-day-old chick embryos is inoculated with *Listeria* strains, is an alternative assay to the mouse pathogenicity test.[489] When a comparison of the two methods was made using 20 *Listeria* strains, a good correlation was found.[381]

Continuous cell lines have also been proposed as a test for pathogenic *Listeria* species. Chinese hamster ovary cells were the most sensitive among eight cell lines, when culture filtrates from *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. murrayi*, and *L. grayi* were tested for a cytotoxic effect.[140] A 6-hour virulence assay was developed using hybridoma and myeloma cells from murine and human sources and evaluated for its usefulness in *in vitro* virulence testing.[38] Three hybridoma cell lines and a murine myeloma line were equally sensitive to virulent *Listeria* species.

44.9 CONTROL

Methods used to control *L. monocytogenes* involve the implementation of basic quality assurance systems such as hazard analysis critical control point (HACCP) procedures from "farm to fork." Many of the problems in food plants linked to final product contamination with *L. monocytogenes* have been due to postprocessing contamination (Figure 44–1). Once the organism contaminates a food processing plant, it can survive there for a long time, especially if the temperature is low and the organism is protected by food components.[390] When suspended in saline or milk, *L. monocytogenes* can survive on fingertips for up to 1 hour and 5 hours, respectively; hand washing with soap usually fails to decontaminate the fingertips.[470] Postpasteurization contamination of food products with listeriae can occur in processing plants via aerosol formation,[201] and the organism can survive for longer than 3 h when present in large numbers in aerosol suspensions.[476] Environmental and in-line sampling have located problem areas and revealed plant conditions that may contribute to final product contamination. With this knowledge in hand, much effort has been expended by the dairy, meat, and fish industries to improve sanitation, hygiene, and general cleanliness inside food processing operations.

44.9.1 Control in the Dairy Industry

General Aspects

Raw milk is contaminated with *L. monocytogenes* either by secretion of the organism directly into the milk by a mastitic cow[197], or an asymptomatic carrier,[150] or by environmental contamination of the udder via soil and/or animal waste in the barn area. A study of more than 8,800 environmental samples from 62 dairy plants revealed that *Listeria* were isolated most frequently from floor drains (38%) and conveyor belts (36%).[369], This is consistent with findings that the organism can be isolated frequently from wet sites within plants, and that fluid milk and plants producing frozen products were more frequently contaminated with the organism than either butter, processed cheese, natural cheese, or dry product plants.[369] The high level of condensate in fluid milk and frozen products plants may play a role in postprocessing contamination. To understand some of the difficulties industry faces in trying to operate in a *Listeria*-free environment, some of the other sites in addition to drains and conveyor belts found to be contaminated with *Listeria* in the 62 dairy plants include: clean-in-place (CIP) rinse tank, case washer water, glycol solution, butter remelt area, return dumping area, hog feed tank drain, roof evaporator drip pan, driveway pit, main entrance, office, and shop.[369] In addition, *Listeria* are frequent contaminants of raw materials used in the plants, and therefore, the organism is continually reintroduced into the plant environment.

Several controls for *Listeria* can be applied at the farm level. A case-control study of 128 selected dairy farms in France revealed that poor silage quality, inadequate frequency of cleaning the exercise area, poor cow cleanliness, insufficient lighting of milking barns and parlors, and incorrect disinfection of towels between milkings were significantly associated with milk contamination by *L. monocytogenes*.[441] The following control measures should be considered.

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- Any cow with slightly hardened quarters should be treated promptly with antibiotics, and areas in the vicinity of aborted cows should be disinfected.
- Mastitic cows should be separated from the rest of the herd.
- Drinking water for cattle should be of good microbiological quality.
- Fields should not be irrigated with effluents from sewage treatment plants or fertilized with untreated sludge from sewage treatment plants.
- Sound milk-collection techniques should be practiced, including teat-dipping and drying and sanitization of all milk contact surfaces just before use.
- The temperature of the bulk-tank should be monitored and kept as low as possible.[82,106,481]

Control at the Dairy Plant

Detailed information is given in several reviews.[82,106,362,366,481] There are four major ways of introducing *L. monocytogenes* into the dairy plant: via contaminated milk, via contaminated materials and equipment, via the environment such as from the air or by pests, or via plant workers, truck drivers, or visitors carrying the organism into the plant. Milk trucks should be properly disinfected, and milk truck drivers should not walk through the production facilities of a plant. Because *L. monocytogenes* can multiply in naturally contaminated raw milk,[150] the temperature in milk silos should be monitored and kept as cold as possible. The following measures can be used to control the organism.

Isolating Processing Areas from Raw Product Areas. Generally, unauthorized personnel should not be allowed into the plant. Traffic patterns must eliminate the potential for cross-contamination (eg, as far as possible, personnel within the plant should not walk from the raw product area to the processing area; for those employees who must do so, footbaths should be installed and managed properly). All outer garments worn in the plant, including boots, should be sanitized daily. Color-coded outer garments should be used to identify the area of the plant to which personnel are assigned. The airflow within a plant should move from finished product area to the outside or to the raw material receiving area. Raw materials should never be stored with finished product. Only those products that have not left the direct control of the dairy should be reworked. Refrigerated nonsterile products should not be returned to the factory when their shelf-life has expired. Reworked products should be set aside in a separate area until they are reprocessed. A general principle is to treat reworked products as raw product. Employee education programs are crucial to success, and must be backed up by the appropriate management action. This would include education of employees on the basics of Good Manufacturing Practice (GMP), provision of the latest technology in terms of sanitation practices, provision of trained supervision, elimination of contamination sources from clean employee environments, and provision of constant feedback and encouragement.

Processing Controls. The proper use and maintenance of pasteurizers is of primary importance. Accurate records on pasteurizer performance must be kept and inspected daily. Sweetwater should be treated with biocide. Air entering a plant should preferably be filtered (gross filters) and the air intake located away from any outside area that may harbor *Listeria*, such as the raw milk receiving or refuse-containing areas. Makeup air should not be taken from dirty areas. Air incorporated into dairy products, such as ice cream, should be filtered through bacterial or high efficiency particulate air (HEPA) filters. Aseptic packaging and processing areas should also be supplied with HEPA-filtered air. All walls, ceilings, and floors must be smooth with no cracks, as *L. monocytogenes* can accumulate in these areas. The processing area should be kept as dry as possible, since *L. monocytogenes* is frequently found in wet areas of the plant.[279] Condensation in processing and packaging areas may be reduced by dehumidifiers and/or air conditioners. Cross-connections between finished and raw product (including CIP lines) should be eliminated. All processing environments should also be kept as cold as possible.

Cleaning and sanitizing. All areas of the plant must be thoroughly cleaned on a regular basis. This should include drains, air handling systems, conveyor belts, cold storage areas, walls, ceilings, floors (including sanitizing floor mats and foot baths), drains, pallets, conveyor belts (tracks), milk cases, case washers, and cold storage areas.[77,279,518] However, priority should be given to product contact surfaces. Of 39 frozen milk plants in California, those plants with above-average sanitation had a much lower rate of recovery of *Listeria* than plants with below average sanitation.[518] In general, sanitation systems must be proven effective with appropriate verification. Cleaning and sanitizing must be given a high priority, as should personnel chosen to oversee the effectiveness of the program. Any spilled dairy products should be wiped up and the immediate area cleaned and sanitized. Items that absorb water, such as rags and sponges, are potential sources of *L. monocytogenes* contamination and should not be reused for cleaning without being sanitized. The use of color-coded brushes for different areas of the plant should be considered. Effective sanitation guidelines for dairy plants have been published.[82,366] Consultation and guidance from the chemical manufacturer are highly recommended. Each processing plant usually has different requirements for cleaning agents, and therefore compound formulations may be designed for each type of cleaning operation. No single hygiene program will fit all the different types of production and processing facilities. In general, on food contact surfaces, listeriae can be inactivated by using sanitizers based on sodium hypochlorite, iodine complexes, quaternary ammonium compounds (QUATs), and anionic acid sanitizers. For nonfood contact surfaces, a combination of thorough cleaning (high pressure, foam, or manual operations) with chlorinated alkaline cleaners, followed by a potable water rinse and a surface sanitizer usually is recommended.[111] Sanitizers are generally more effective against *L. monocytogenes* that are attached to nonporous rather than porous surfaces.[324] It is generally recognized that to properly control listeriae, sanitation programs must go well beyond those previously thought to be adequate.

Environmental and Product Sampling. Environmental sampling should be done to test the efficacy of the sanitation program and also to pinpoint possible "hot spots" in the plant where *L. monocytogenes* may colonize.[178,522] Drains are good indicator sites of the environmental status of the plant.[77] Guidelines both for taking and analyzing environmental swabs and in-line samples for the presence of *L. monocytogenes* in meat plants have been published.[178] Testing for *L. monocytogenes* in the finished product as the sole means of control is not an efficient way of ensuring a safe product. The HACCP system now being implemented by industry uses finished product testing only as a means of verifying that GMPs are in place.

44.9.2 Control in the Meat Industry

Many of the control points outlined in the section on the dairy industry also apply to the meat and seafood industries. Both the Canadian government and the American Meat Institute have published information handbooks on the control of *L. monocytogenes* in RTE meat products.[8,178] Some of the places where *L. monocytogenes* has been found in meat plants, listed approximately in the order of prevalence, are floors, drains, sponges and brushes, food contact surfaces, condensate, walls and ceilings, and compressed air.[28] Other potential problem sites include conveyor belts, transport containers, cutting tables, equipment and boards, air above fans, air conditioners, humidifiers, employees' clothing and hands,[186,187] exhaust hoods, gutters, and pickling systems.[8,186,522] Control can require expensive modifications of existing plant infrastructure or equipment.

Because it is nearly impossible to eliminate *L. monocytogenes* from raw meat, most control procedures aim at minimizing levels in raw meat, avoiding cross-contamination of processed product, preventing multiplication of the organism in products, and preventing its establishment and growth in the environment of the meat plant.[497] There is also a need for increased attention and education for retail operators, especially those with delicatessen counters.[3] *L. monocytogenes* was isolated from the hands of 3 of 10 delicatessen workers from four different establishments.[275] Cross-contamination of foods has occurred in retail outlets.[3,252] For example, if soft-cheese wheels are cut at the retail level by a single knife, contamination of one wheel could result in cross-contamination of many other cheeses that are cut by the same knife. On the cheese surface, the organism could multiply to high numbers during storage on the retail shelves and in the consumer's refrigerator.[17,188] This can also occur with meats cut by a retail meat slicer.[243] Measures aimed at control include:[8,178,497,498]

- improvement of slaughterhouse hygiene
- separation of raw meat and thermally processed meat areas
- regular, proper, and effective cleaning and sanitation programs
- disinfection of hands, especially in the processing and postprocessing areas, with regular changing of gloves[186,187]
- pressure hoses not being used to clean drains, since resulting aerosols can spread contamination throughout the room
- identifying and establishing procedures to deal with possible high-risk circumstances (eg, introduction of new product onto line)
- no direct access from outside the plant to the production and packaging areas for personnel or equipment such as forklifts

- effective processing schedules with immediate cooling
- recontamination of cooked meat products should be avoided and the use of cook-in-the-bag processing or immediate aseptic packaging of finished product encouraged[261]
- maintenance of a dry environment within the meat plant
- commitment of management to provide education and training to plant personnel
- establishment of an effective environmental sampling program to verify the effectiveness of the control programs, with routine sampling of environmental surfaces
- the use of "clean rooms" for meat plants designed to prevent recontamination of heated meat products should be considered and a "clean room mentality" established within the RTE product area.[28,299]

Unless properly applied, cleaning and disinfection procedures will not minimize the levels of this organism, leading to loss of control.[440,498] Floors are particularly difficult areas to maintain free of *Listeria*, especially during the summertime. Equipment should be designed and constructed so that it is easily cleaned, and without corners. It may take from 6 months to 2 years to bring a plant under control.[498]

Environmental sampling of a turkey frankfurter plant, whose product had been linked to a case of listeriosis, showed that contamination of the majority of frankfurters occurred at a single point during the peeling process, just before packaging.[522] A properly applied HACCP-based sampling plan may have pinpointed this process in the plant, and thus eliminated contamination of the final product. In addition to the use of HACCP, research is continuing on the use of additional hurdles or barriers to control the growth of *L. monocytogenes* in meat and poultry products. This would include the addition of chemical compounds and/or the use of starter cultures such as the lactic acid bacteria and their bacteriocins.

44.9.3 Compounds Used To Inhibit or Inactivate *L. monocytogenes*

Compounds that are used most commonly by the food industry and that are effective against *L. monocytogenes* include iodophors such as iodine (25 ppm); acid sanitizers (130 to 200 ppm); chlorine at a wide range of concentrations (20 ppm free available chlorine for water treatment; 200 ppm for CIP cleaning, walls, stainless steel equipment, and nonporous surfaces; and 1,000 ppm for wooden crates and fogging of the atmosphere) and QUATs at 200 ppm.[85,129,310,368,500] A new buffered organic acid anionic surfactant was also effective (> 5 log kill after 30 seconds) against *L. monocytogenes* at a concentration of 0.6%.[423]

The presence of organic material and the attachment of microorganisms to surfaces can reduce the efficacy of disinfectants against *L. monocytogenes*.[32,368] Bacteria within a biofilm are generally more resistant to physical and chemical antimicrobial factors than are freely suspended bacteria.[168,296] On surfaces that are irregular or difficult to reach, survivors might form biofilms, resulting in increased resistance to sanitizers and heat and they could then contaminate foods.

44.9.4 Use of the Hazard Analysis Critical Control Point System

HACCP procedures should be applied during production of foods, at the retail level, and in the home. Because *L. monocytogenes* can grow at chill temperatures, a product that may have contained very low numbers immediately after leaving the processing plant could contain large numbers by the time the food is consumed. This is especially true in the case of the new generation refrigerated-type foods with extended shelf-life. For these foods, it is necessary to add additional barriers or hurdles to control the growth of *L. monocytogenes*. Controls at both the retail and consumer levels include prevention of cross-contamination and maintenance of chill cabinets and refrigerators at as low a temperature as possible.

44.9.5 Regulatory Policies

Unfortunately, control measures for *L. monocytogenes* in foods are also influenced by differing worldwide government policies regarding the presence of the organism in food—a problem compounded by the lack of knowledge regarding the numbers of cells that must be ingested to cause illness.[230,231] Whether or not food regulatory agencies worldwide should tolerate low levels of *L. monocytogenes* in foods in which the organism cannot grow is still debatable at this point. However, every effort should be made to inhibit multiplication of the organism in foods. It has been suggested that all strains of *L. monocytogenes* should be regarded as potentially pathogenic.[342] Despite the unknowns, the HACCP approach is the most reasonable path to follow to reduce and/or eliminate *L. monocytogenes* in foods. The *L. monocytogenes* policies of various countries are outlined briefly below.

North America

Following the large listeriosis outbreak in 1985 that was clearly linked to the consumption of Jalisco cheese,[306] a "zero tolerance" policy was instituted that required an absence of *L. monocytogenes* in 50g of food. Despite subsequent debate, this policy has not been altered;[323,347] however, the number of listeriosis cases and deaths in the US for the years 1989 to 1993 decreased by 44% and 48%, respectively.[486] The decreased rates were seen in both the perinatal and nonperinatal populations. An outbreak of listeriosis in the US between August 1998 and February 1999, which has affected at least 70 people and caused 20 deaths, is thought to be associated with the consumption of hot dogs and deli meats.[74a] This outbreak, which occurred after it was thought that industry had brought the problem under control, has caused renewed concern about *L. monocytogenes* in the US.

Canada, meanwhile, had the opportunity to develop a compliance guide in the absence of a crisis situation, which led to a flexible policy.[146] The Canadian regulatory policy on *Listeria*-contaminated foods, based on the principles of HACCP, was developed using a health risk-assessment approach.[146] The policy reflects current knowledge that the risk of contamination by *L. monocytogenes* can be reduced, but the organism cannot always be eradicated from finished product or the environment. The policy directs inspection and compliance action to RTE foods that are capable of supporting growth of the organism. The highest priority is given to those RTE foods that have been linked causally to listeriosis and those with a shelf-life greater than 10 days. The policy is based on a combination of inspection, environmental sampling, and product testing. Foods have been placed into three categories based on health risk. Products in Category 1 have been linked causally to outbreaks of listeriosis and receive the highest priority in inspection and compliance activities. The presence of *L. monocytogenes* in these RTE foods will trigger a Class I recall with consideration of a public alert. Category 2 contains all other RTE foods that are capable of supporting growth of *L. monocytogenes* and have a shelf-life exceeding 10 days. The presence of *L. monocytogenes* in these products requires a Class II recall with possible consideration of a public alert. Category 3 contains two types of RTE food products: those supporting growth with a ≤ 10-day shelf-life and those not supporting growth. For Category 3 RTE foods, factors such as the presence or absence of GMP, levels of *L. monocytogenes* in the food (action level 100 cfu g^{-1}), and/or a health hazard evaluation are all considered in the compliance action taken.

Europe

The UK government only has specific legislation for *L. monocytogenes* in cheese and other milk-based products[11] and is now required to put into the UK food regulations, microbiological standards included in Directives issued by the European Community (EC). The EC directive on milk and milk-based products, which is the same as the UK legislation, states that *L. monocytogenes* should not be present in 25g ($n = 5$, $c = 0$) of soft cheese and should be absent in 1g of other products.[341] In addition, the Public Health Laboratory Services (PHLS) has established provisional guidelines for some cooked RTE foods at point of sale.[191] The four categories developed along with their action levels include: (1) satisfactory (*L. monocytogenes* not detected in 25g), (2) fairly satisfactory (present in 25g at levels of <100 cfu g^{-1}), (3) unsatisfactory (100 to 1000 cfu g^{-1}), and (4) unacceptable/potentially hazardous (> 1000 cfu g^{-1}).

Germany has developed recommendations to be used by food inspection authorities.[490] Four different categories of food have been developed along with action levels. Group I consists of foods for infants and small children as well as dietary foods, with the action level being the presence of *L. monocytogenes* in 25g of product. Group II contains foods such as pasteurized milk and aseptically packaged products, with the same action level as Group I foods. Foods in Group III include frozen meals, cheese (but not raw-milk cheese), prepacked sausages and shrimp, with an action level of >100 cfu g^{-1}. Low-level contamination would precipitate follow-up action in the plant, and higher contamination would render a food "unfit for human consumption." The last group (Group IV) consists of three different food categories that are "otherwise stabilized foods," such as smoked fish and fermented sausages; raw foods consumed in the raw or unprocessed state; and raw food that is heated before consumption. For Group IV foods, *L. monocytogenes* levels of < 100 cfu g^{-1} usually are permissible, but in cases where higher levels are observed, the food plant or shop would be inspected. The upper limit of *L. monocytogenes* allowable in any food product is currently 10^4 cfu g^{-1} or ml^{-1} (action level, not tolerance value), but authorities are considering lowering this to 10^3 cfu g^{-1} or ml^{-1}.[490]

Australia

Standards limiting *L. monocytogenes* contamination of foods are not yet incorporated into the Australian Food Standards Code. State government agencies will intervene to prevent the sale of foods contaminated with the organism;[132] however, different policies and attitudes prevail in the various state agencies, creating some confusion. Export control orders are more stringent than the Foods Standards Code and already require the absence of *L. monocytogenes* from 5 × 25g samples of many cheeses. The National Food Authority is considering the introduction of standards for the organism into the Food Standards Code. Although the Authority previously rejected a zero-tolerance approach for the organism in liquid and powdered milk as well as cream, it is currently considering adopting a zero-tolerance policy (absence in 5 × 25g) for foods associated with *Listeria* outbreaks and foods capable of supporting growth of the organism.[132] As in the Canadian approach, products will be grouped on the basis of ability to support the growth of *L. monocytogenes*, and information from predictive models will be used.

The International Commission on Microbiological Specifications for Foods (ICMSF) has developed a policy mainly for the international trade of foods containing *L. monocytogenes.*[246] Cases and sampling plans for the organism were based on principles published previously.[245] A decision tree (divided into four steps) for the choice of cause for *L. monocytogenes* is presented. Step I asks if the food is intended for highly susceptible individuals; step II if the food has been epidemiologically linked with listeriosis; step III if the food has a history of high levels of the organism, and questions its potential for supporting growth of the organism; and step IV if the food will receive a listericidal treatment before consumption. For food intended for normal individuals, the ICMSF recommends, n (ie, number of samples) values of 5, 10, or 20 with a small m of ≤ 100 cfu g^{-1}, and for food intended for highly susceptible individuals n values of 15, 30, or 60 with a small m of zero in 25g. The requirement of ≤ 100 cfu g^{-1} of food applies at the point of consumption.[247]

In addition to these policies, each country listed above has published educational brochures and pamphlets targeting the high-risk populations. For example, in Australia, billboards have been placed on the back of city buses warning pregnant women about the risk of foodborne listeriosis. Dietary guidelines have also been published by the respective government agencies; these include advice on safe food handling, and advice to high-risk groups to avoid the consumption of soft cheese and pâté.

44.10 CONCLUDING REMARKS

Although listeriosis has been described for many years, the demonstration of foodborne transmission of the organism has made it a major concern to the food industry. *L. monocytogenes* can contaminate nearly all kinds of food and can grow on many foods even at refrigerator temperatures. Methods for isolating *Listeria* have been developed, although comparative studies have shown that no one procedure gives consistent isolation from all contaminated samples. Optimization of rapid detection methods for this bacterium in foods is now required.

The virulence factors of *L. monocytogenes* have been identified genetically, allowing the design of rapid methods to detect the organism in foods. Molecular typing of wild strains demonstrates the heterogeneity of virulence within the species, and the study of epidemic strains shows a low number of closely related clones. Perhaps the epidemic strains share one or a few specific properties that could explain their causing large outbreaks.

Surveillance systems should be initiated so that time-space clusters can be detected, and common source outbreaks identified, investigated, and prevented. They could also help to explore the differences in reported incidences, which may be due to environmental factors, host susceptibility, or dietary exposures. Infections caused by contaminated foods need to be more precisely defined by epidemiological investigations, including strain typing.

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45 Salmonella

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45.1 INTRODUCTION

From the early recognition of *Salmonella* spp. as the etiological agent for human typhoid fever and cholera in swine,[88,403,578] public health and socioeconomic concerns on the occurrence of these bacteria in the global food supply progressively led to the identification of *Salmonella* reservoirs, to the characterization of epizootic and zoonotic cycles of infection, and to the human health impact arising from the massive production and marketing of foods for national and international markets. Historically, the international trade of agricultural and food products contributed to the introduction and spread of new *Salmonella* strains in importing countries. For example, the emergence of *Salmonella* Agona in the United States (US) and the United Kingdom (UK) nearly three decades ago stemmed from the importation of contaminated Peruvian fish meal.[409,451,608] The microorganism, which rapidly spread within the meat animal industry and in human populations, continues to figure in the national epidemiological registries of these countries.[69] Imported animal feeds were also incriminated as the source of *S.* Hadar in turkey flocks, and of *S.* Indiana and *S.* Saint-paul in the poultry industry of the UK between 1959 and 1961.[409] During World War II, contaminated dried eggs imported into the UK from North and South America were diverted principally to the swine industry as feedstuff. This practice reportedly introduced *S.* Panama into the human food chain of the importing country and led to numerous episodes of human salmonellosis.[409] The widespread occurrence of salmonellae in internationally distributed animal feeds and feed ingredients has been, and undoubtedly continues to be, responsible for the emergence of novel *Salmonella* serovars in meat products and in human foodborne disease.

Recent decades have also witnessed the emergence of new foods as sources of human *Salmonella* infections. Chocolate, which was generally considered to be a safe food product, was implicated in a major Canada-US outbreak of *S.* Eastbourne in 1973. Cocoa beans presumably imported from Africa were tentatively identified as the source of contamination.[177] Several episodes of human salmonellosis from the consumption of internationally distributed chocolate products were reported in subsequent years.[164] In the past, limited survey data suggested a low prevalence of *Salmonella* spp. in fresh fruits and vegetables, and occasional episodes of human salmonellosis were attributed to these products.[164] Although melons, notably watermelons and cantaloupes, were primarily responsible for the isolated fruitborne outbreaks from 1950 to 1991, the consumption of other products such as tomatoes and orange juice has resulted in cases of human salmonellosis. Similarly, the historical prominence of spices as a potential source of human salmonellosis is currently being overshadowed by the growing importance and disease potential of other vegetables, notably leguminous sprouts.[166]

Poultry, pork, and beef products, in decreasing order of importance, continue to figure prominently as the principal reservoirs of human foodborne salmonellosis. Efforts to control the spread of salmonellae in the rearing and slaughtering sectors have met with limited success due, in part, to the uncoordinated implementation of control measures in these vertically integrated industries.

Although S. Typhimurium has consistently headed national listings of serovars implicated in human disease before 1985, its dominance was challenged by S. Enteritidis, which now prevails in many countries as the most important disease-associated serovar.[160,705] The association of S. Enteritidis with poultry products, notably shell eggs, contributed significantly to this phenomenon of serovar succession.[4,11,447] Early reports on the pathogenicity of *Salmonella* indicated that ingestion of high numbers (10^5 to 10^7) of foodborne salmonellae was a prerequisite for human illness.[164] However, more recent evidence suggests that a single *Salmonella* cell may constitute a human infectious dose.[166] Major efforts to educate consumers and workers in food service establishments continue to yield disappointing results as evidenced by the persistence of improper handling and inadequate cooking of foods as the principal causes of bacterial foodborne outbreaks.[653]

This chapter assesses the magnitude of the *Salmonella* problem in the global food chain based on epidemiological, pathogenic, and ecological considerations and underlines the role of control measures at all levels of food production, manufacture, and distribution and of reliable analytical methods as prerequisites to the safety of the food supply.

45.2 TAXONOMY AND CHARACTERISTICS OF THE ORGANISM

Salmonella spp. are facultatively anaerobic Gram-negative (GN) rods within the family *Enterobacteriaceae*. Members of this genus are motile by peritrichous flagellation except S. Pullorum and S. Gallinarum, which lack flagella.[39] Nonmotile variants can also arise as a result of a faulty assembly of flagellar subunits or deficiencies in the motor functions of these appendages.[432] Salmonellae grow optimally at 35° C to 37° C, catabolize a variety of carbohydrates into acids and gas, use citrate as the sole carbon source, produce H_2S, and decarboxylate lysine and ornithine to cadaverine and putrescine, respectively. These microorganisms are oxidase-negative and catalase-positive with a 50 to 53 mole % guanine plus cytosine (G + C) DNA content.[404] Historically, an isolate from selective plating media was most likely to be confirmed as *Salmonella* if it catabolized glucose and lysine but failed to metabolize lactose, sucrose, and urea. The reliability of these diagnostic traits is being undermined by the widespread exchange of genetic elements between compatible bacterial strains in the environment.[278,364] Atypical *Salmonella* biotypes that cannot decarboxylate lysine[325,400,472] or that readily use lactose,[278,364,429,571,646,661] sucrose,[364,520,549] and urea[232,374] have been isolated.

Several schemes based on biochemical characteristics, DNA homology, and enzyme electrophoretic patterns have been used for the taxonomic classification of salmonellae. In 1966, Kauffmann used determinant biochemical traits to separate the *Salmonella* group into four subgenera (I to IV) with each serovar given species status.[381] A fifth subgenus (V) was subsequently recognized.[404] The main characteristics of each subgenus included the ability to use dulcitol (subgenus I), dulcitol and malonate (subgenus II), malonate and o-nitrophenyl-β-D-galactopyranoside (ONPG) (subgenus III), growth in KCN (subgenus IV), and failure to metabolize dulcitol and malonate and ability to catabolize ONPG and grow in KCN (subgenus V). In 1972, Ewing proposed another taxonomic scheme that recognized only three species within the *Salmonella* group. These included S. Typhi (single serovar), S. Choleraesuis (single serovar), and S. Enteritidis, which encompassed all of the remaining serovars. Members of the *Arizona* group were assigned to a distinct *Arizona* genus.[229] This scheme was structured on the differential utilization of 15 substrates and production of hydrogen sulfide gas. A third taxonomic scheme based on phenetic and DNA homology characteristics recognized S. Choleraesuis as the sole species subdivided into seven subspecies including subspecies *choleraesuis*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *bongori*, and *indica*.[407] In 1987, the latter scheme was modified to recognize *S. enterica* as the sole species consisting of the seven foregoing subspecies with a change of subspecies *choleraesuis* to subspecies *enterica*.[406] The type strain was also changed from *S. choleraesuis* to *S. enterica* subspecies *enterica* serovar Typhimurium LT 2. In 1989, the subspecies *bongori* was elevated to species level based on discriminating multilocus enzyme electrophoretic patterns.[548] The *Salmonella* genus presently consists of two species: *S. enterica* including six subspecies (*enterica, salamae, arizonae, diarizonae, houtenae,* and *indica*) and *S. bongori*. No fewer than 2,422 serovars are recognized within the genus (Table 45–1). All name-bearing serovars belong to subspecies *enterica* whereas serovars in the remaining subspecies of *S. enterica* and in *S. bongori* are identified by antigenic formulae.

45.3 CHARACTERISTICS OF DISEASE

45.3.1 Clinical Symptoms and Treatment

The clinical presentation of human salmonellosis may correspond to either the enteric fever syndrome following infection with typhoid or paratyphoid strains or to the nontyphoid-dependent gastroenteritis/enterocolitis with possible progression to a more serious systemic infection. Humans are particularly vulnerable to *S.* Typhi and *S.* Paratyphi A, B, and C infections because of the ability of these strains to invade and multiply within host tissues. Enteric fever can result from exposure to an infected individual or from contact with contaminated food and water. Clinical symptoms appear 7 to 28 days following exposure to these serovars. Watery diarrhea or, infrequently, constipation, persistent and spiking fever, abdominal pain, headache, nausea, prostration, and a rash of rose spots on the shoulders, thorax, or abdomen may occur. Complications of enteric fever include intestinal bleeding from ileal ulcers or intestinal perforation resulting from hyperplasia of the epitheliolymphoid Peyer's patches. Clinical diagnosis of the disease commonly is based on symptomology and isolation of the causative agent from blood samples during the first 2 weeks of illness. Stool and urine samples are less dependable. Isolation from bone marrow can be valuable, particularly with patients who are already undergoing antibiotic treatment.[111,440,711] Enteric fever may be identified by the Widal immunological test whereby antibodies in the sera of patients agglutinate with *S.* Typhi somatic and flagellar antigens.[131] Enzyme-linked immunosorbent assays (ELISA) for the detection of Vi- or lipopolysaccharide-specific antibodies in human serum have also been proposed.[440] Encouraging results were recently obtained with a Vi antigen-specific DNA probe for the detection of *S.* Typhi in infected blood.[569] Clinical management of human typhoid and paratyphoid infections rests on an aggressive course of antibiotic therapy. Although chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole (SXT) once constituted the mainstay of treatment, the emergence and persistence of strains resistant to these drugs notably in Asia and Central and South America[515,563,568,711] has resulted in the use of cephalosporins and fluoroquinolones.[48,285,442,563,617] Between 1.0% and 5.0% of typhoid patients become chronic fecal carriers after the remission of clinical symptoms,[111,285] but prolonged treatment with fluoroquinolones such as ciprofloxacin and norfloxacin can significantly reduce the rate of chronic carriage.[48,285]

Table 45–1 Distribution of Serovars within the *Salmonella* Genus[523]

Species	*Subspecies*	*Number of Serovars*
S. enterica	*enterica*	1,427
	salamae	482
	arizonae	94
	diarizonae	319
	houtenae	69
	indica	11
S. bongori		20
Total		2,422

Source: Data from M.Y. Popoff, J. Bockemuhl, and F.W. Hickman-Brenner, Supplement 1995, No. 39, to the Kauffmann-Wite Scheme, *Research in Microbiology*, Vol. 147, pp. 765–769, © 1996.

The symptoms of nontyphoid salmonellosis may include nausea, abdominal cramps, diarrhea with watery and possibly mucoid stools tinged with blood, fever of short (<48 hours) duration, and vomiting that appear 8 to 72 hours following exposure to the bacterium.[333,711] Isolation of the causative agent from stools or other infected sites confirms salmonellosis. The treatment of *Salmonella* enterocolitis with antibiotics generally is contraindicated because it tends to prolong the excretion of the microorganism in stools and does not alter the normal course of the disease. Fluid and electrolyte replacement may be indicated for severe cases of diarrhea. The clinical symptoms of uncomplicated enterocolitis usually subside within 5 days followed by a convalescent carrier state of one to several months during which the asymptomatic individual continues to excrete salmonellae. Personal hygiene during this period is critical if cross-infection in the home or workplace is to be avoided. Enterocolitis does not lead to acquired immunity and re-exposure to nontyphoid salmonellae will most likely result in disease. Nontyphoid salmonellosis can progress from an enterocolitic to a septicemic condition because of the migration of salmonellae from the intestinal tract into deeper tissues through vascular and lymphatic conduits. Although stool cultures generally are negative at this stage, blood samples will frequently yield salmonellae. Systemic spread of the pathogen can lead to cardiac and circulatory anomalies, intracranial infection, and dysfunction of major body organs.[135,182,299,383,562] Fluoroquinolones are being used increasingly for the treatment of septicemic conditions.[48,392,530,587] Several important chronic sequelae can also be associated with nontyphoid salmonellosis and other enteric infections (Chapter 55). These include arthropathies such as aseptic reactive arthritis (RA), Reiter's syndrome, (RS), ankylosing spondylitis (AS), and rheumatoid arthritis as well as human deficiencies in nutrition and intestinal absorption.[42] There is an apparent genetic predisposition of individuals carrying the human leucocyte antigen (HLA) B27 gene within the chromosomal major histocompatibility complex (MHC) class I locus to the development of RA, RS, or AS several weeks after the acute phase of nontyphoidal salmonellosis.[107,581,615,644] The RA and RS conditions share clinical features such as inflammation of articulated joints of upper and lower limbs, conjunctivitis, and urethritis and frequently progress to AS as evidenced by the inflammation of vertebrae.[107,615] These pathologies develop and persist in the absence of an active *Salmonella* infection and in the absence of viable microorganisms in the inflamed anatomical regions. One hypothesis concerning the relationship between the predisposing human HLA-B27 genotype and the host-pathogen interactions culminating in arthritides suggests a cross-reactivity of antibody to *Salmonella* antigen(s) and HLA-B27 antigens located on the surface of host nucleated cells. Following the immunological clearance of the invasive salmonellae, circulating antibodies specific to the infectious strain bind to the human HLA-B27 antigen. This leads to an arthritic autoimmune disorder in which reactions between synovial T-lymphocytes and HLA-B27 antigen-antibody complexes produce chronic inflammation of the affected joints.[107,615] The ability of antibodies to nonarthritogenic bacteria to recognize the HLA-B27 antigen questions the reliability of the cross-reactivity hypothesis. Another putative mechanism relates to the ability of salmonellae to alter the host HLA-B27 antigen to a state that host T-lymphocytes will recognize as a foreign antigen, thereby triggering an arthritic inflammatory response.[262] The failure of other workers to corroborate these preliminary experimental findings undermines the acceptance of this theory.[107] Other HLA-B27 independent mechanisms for RA have been proposed. The chemical degradation and release of *Salmonella* lipopolysaccharides (LPS) by host phagocytes followed by the incorporation of the degraded LPS into host cell membranes could potentiate an autoimmune response involving LPS-presenting host cells and natural immunological processes.[107,287] Stress proteins produced by bacterial and host cells as a protective response to adverse environmental conditions are highly conserved[261,615,696,712] and may be involved in the induction of reactive arthritides. This suggests that the activation of T-lymphocytes against *Salmonella* stress proteins could trigger a concurrent autoimmune response from a lymphocyte recognition of host stress proteins on the surface of synovial cells. Contradictory reports on the importance of stress proteins in the development of RA underline the need for further research in this area of *Salmonella* pathogenesis.

45.3.2 Prophylactics

The development of human parenteral heat-inactivated, phenol-preserved whole cell typhoid and paratyphoid vaccines at the close of the nineteenth century contributed significantly to the reduction of enteric fever in endemic areas.[414,440] These vaccines confer 80% to 90% protection for 3 to 7 years and are associated with low to moderate levels of adverse reactions. The preparations stimulate serum antibody production but are poor inducers of cell-mediated and local secretory immune (s IgA) responses.[675] An oral typhoid vaccine manufactured by the Swiss Serum and Vaccine Institute (Berne) under the trade name Vivotif Berna consists of three enteric-coated capsules each taken on alternate days. The treatment provides 60% to 80% protection for up to 7 years with booster doses recommended at 3-year intervals.[675] The vaccine *S.* Typhi Ty21a strain was originally derived from random mutagenesis with nitrosoguanidine and ultraviolet light irradiation.[267] The mutant lacks the Vi surface antigen and contains a mutation in the galactose epimerase (*gal E*) locus, which prevents the synthesis of complete LPS.[122,210] The latter condition results in a rough variant that is highly susceptible to lysis by the host complement system.[162] Although the *gal E* mutation putatively formed the basis for strain attenuation, subsequent studies failed to support this hypothesis.[112] The Ty21a strain harbors a mutation in the genomic *rpoS* locus that normally encodes protective proteins under environmental stress conditions and that likely contributes to strain attenuation.[560] Another recently marketed typhoid vaccine uses the Vi capsular polysaccharide as immunogen. This injectable preparation is distributed by the Pasteur Mérieux Sérums et Vaccins (Lyon, France) under the trade name Typhim Vi. A single dose of this phenol-preserved antigen produces low to moderate levels of secondary reactions and confers protection for up to 3 years.[382]

The clinical success of the oral Ty21a and the injectable Vi vaccines has not dampened research interests in the development of novel typhoid and paratyphoid vaccines providing high levels of immunogenicity, biological safety, and storage stability and engendering few side effects. The use of attenuated strains of *S.* Typhi as potential immunogens has been investigated because of the ability of viable but avirulent and nonreverting typhoid mutant strains to induce strong humoral and cell-mediated responses. Auxotrophic mutant strains that are blocked in essential biosynthetic pathways (*aro*, *pur*, *his*) or typhoid mutants that are deficient for adenylate cyclase and the cyclic adenosine monophosphate (cAMP) receptor protein (*cya*, *crp*) are promising genetically defined variants.[112,122,153,154] The recently characterized typhoid strain incapable of growth at temperatures greater than 29° C may prove useful as an oral vaccine.[66] The favorable evaluation in humans of an oral typhoid vaccine based on an *S.* Typhi Ty2 strain harboring deletion mutations in *aro C* and *aro D* (designated CVD 908) may culminate in the marketing of a novel single-dose oral typhoid vaccine.[332] An injectable semisynthetic Vi vaccine has been synthesized consisting of vegetal pectin acetylated in the C2 and C3 positions and conjugated to tetanus toxoid.[630] The acetylated pectin is a lower molecular weight homolog of the Vi antigen and is nonimmunogenic in mice. The injection of mice with one or two doses of tetanus-pectin conjugate produced greater serum levels of Vi-specific antibodies than those obtained with purified Vi antigen alone. Future trials on the efficacy of conjugated pectin as a parenteral typhoid vaccine clearly are indicated. Although highly desirable, the development of nonviable *Salmonella* oral vaccines has met with limited success because of a need for oral immunogens that resist degradation by gastric acidity and digestive enzymes and trigger a specific immune response at the surface of the intestinal mucosa.[210] Encapsulation of immunogens in acid-resistant and biodegradable materials may resolve this problem.[485] Notwithstanding the success of nonviable oral vaccines against cholera and *E. coli* enterotoxins in stimulating mucosal immunogenicity, a similar *Salmonella* vaccine has not yet been developed.[210]

There is interest in the development of nontyphoidal vaccines for humans and farm animals. The great diversity and rapid temporal succession of serovars in the environment and in the global food chain, and the heterogeneity of immunogenic bacterial surface organelles continue to hamper the development of effective *Salmonella* vaccines. Live vaccine constructs are based on genetic mutations that render viable salmonellae avirulent without adversely affecting their immunogenicity. Although the *gal E* mutation successfully attenuated *S.* Typhimurium and *S.* Enteritidis, it failed to curtail the virulence of *S.* Typhi and *S.* Choleraesuis.[154,331] Clearly, the use of the *gal E* lesion alone is of limited value in vaccine development. Strain attenuation by deletion mutations in the purine (*pur*) genes led to similarly unfavorable conclusions.[114] Transposon mutagenesis in *aro* genes of the aromatic pathway results in auxotrophy and strain attenuation resulting from an inability to synthesize essential compounds such as p-aminobenzoic and dihydroxybenzoic acid, precursors of folic acid and the iron chelating enterochelin, respectively. The nonavailability of these metabolic precursors in host tissues prevents the proliferation of the autotrophic mutant in host cells. *Aro* mutants have found wide application in oral and parenteral vaccines for humans and animals including cattle, sheep, and poultry.[122,138,154,613] Deletions in the adenyl cyclase (*cya*) and the cAMP receptor protein (*crp*) genes lead to attenuated strains that trigger strong and durable immunological responses in orally vaccinated animal models.[154] Mutant strains harboring a single deletion in *cya* or *crp* can revert to the parent genotype, whereas strains with double deletions are quite stable.[114] The vaccine potential of other mutations such as deletion in the *pho P* and *pho Q* genes encoding protective proteins for the survival of *Salmonella* in host macrophages, deletion in the *cdt* locus that potentiates bacterial invasion of deep tissues, curing of virulence (*spv*) plasmids, and lesions in the outer membrane protein regulator (*omp R*) gene continue to attract research interest.[59,114,122,154,210]

Another important facet of vaccine development has been the construction of viable and attenuated *Salmonella* mutants for the delivery of heterologous antigens. Prerequisites for effective and safe recombinant *Salmonella* vectors include avirulence, strong immunogenicity, and a minimum of two stable, nonreverting attenuating deletion mutations.

Although plasmid vectors carrying a foreign gene have figured prominently in developmental research on hybrid *Salmonella* vaccines, problems of vector stability in carrier strains have shifted research interests toward a more stable incorporation of foreign antigens into the *Salmonella* chromosome.[122] However, the latter approach suffers from a potentially low immunogenic response owing to the insertion of a single gene copy into the *Salmonella* chromosome. This is in sharp contrast to the use of multiple plasmid-inserted gene copies in a single *Salmonella* carrier cell. Notable developments have included a bivalent oral typhoid-tetanus vaccine derived from the insertion of the tetanus fragment C toxin gene into the chromosome of a live attenuated *aro A* and *aro C* strain of *S.* Typhi Ty2. Preliminary human trials with this preparation are imminent. The same research group is also developing a more potent human typhoid-tetanus oral vaccine wherein the fragment C gene is carried on a plasmid vector whose expression is under the control of an inducible *nir B* promoter that not only confers stability to the plasmid vector, but also directs a high level expression of the tetanus toxin gene.[122] Attenuated typhoid and nontyphoid vaccines have been developed also for the delivery of foreign antigens such as the form I somatic antigen of *Shigella sonnei*,[666] the binding subunit of the heat-labile toxin (LT-B) of enterotoxigenic *Escherichia coli*,[132] the somatic antigen of *Vibrio cholerae* serotype Inaba,[631] and the hepatitis B and papillomavirus specific antigens[395,588] to mammalian immune systems.

45.3.3 Incidence of Human Salmonellosis

Recent reports from international sources continue to underline the importance of *Salmonella* spp. in human diarrheal disease and in focal and systemic infections. Contributing factors may include contact with an infected individual, exposure to a contaminated environment, or the ingestion of foods containing this microorganism.[162,166] The reported incidence of human salmonellosis varies widely among countries [Table 42–2] and likely stems from the endemicity of the disease in certain countries and from inequalities of national health reporting systems and in the containment of outbreaks. Dramatic increases in the reported rates of human *Salmonella* infections in continental Europe, notably Denmark, Italy, and Lithuania, are disquieting. The reason(s) for the unusually high rate of human *Salmonella* infections in the former Czech Republic are uncertain.

Table 45–2 Reported Incidence of Human Salmonellosis

Country	*Period*	*Rate**	*Reference*
Austria	1989	62.0	266
Bulgaria	1992	25.2	705
Canada	1993	29.7	308
Former Czech Republic	1992	422.0	705
Denmark	1985	27.0	705
	1989	69.0	705
	1995	70.3	34
England and Wales	1994	60.8	28
Finland	1993	56.0	705
Germany	1989	103.0	266
	1991	180.0	589
	1992	243.0	266
	1993	175.5	705
Hong Kong	1993	40.0	699
Hungary	1992	122.3	705
Iceland	1993	31.9	705
Israel	1983	63.0	594
	1991	85.0	380
	1992	100.9	705
Italy	1988	19.5	705
	1992	41.3	705
Lithuania	1988	14.7	705
	1992	72.1	705
Poland	1989	80.0	265
	1993	77.0	705
Scotland	1985	32.0	601
	1993	58.0	601
Slovakia Republic	1985	118.0	266
	1992	187.0	705
Sweden	1980–83	32.2	692
	1991	69.0	13
Switzerland	1989	105.0	266
	1992	119.0	705
	1993	89.0	705
United States	1995	17.7	30
Former Soviet Union	1989	55.0	266

* Rate per 100,000 population.

Newborns, the elderly, and immunocompromised individuals are particularly susceptible to bacterial infections in general and to salmonellae in particular.[30,205,705] Hospital nurseries and neonatal intensive care units are particularly vulnerable to the rapid spread of *Salmonella* infection following the admission of an infected infant.[46,301,457,626] Infections can develop from the frequent contact between the nursing staff and the patients, invasive clinical procedures such as catheterization, intubation, and thermometry, and from the creation of aerosols in poorly ventilated hospital units.[626] Nursing homes and geriatric hospital wards are equally prone to the rapid spread of *Salmonella* among residents and support staff, with a propensity for clinical complications and death of elderly patients.[149,370,621] Although young and mature adults are more resistant to *Salmonella* infections, strain virulence, infectious dose, underlying ailments, prophylactic or therapeutic drug treatments before exposure to invasive salmonellae, achlorhydia arising from gastric resection or metabolic dysfunction, and the nature of an ingested contaminated food can facilitate the entry and persistence of the bacterium in the gastrointestinal tract.[160,166] The view that all *Salmonella* serovars are potentially pathogenic to man is reinforced by reports of disease from members of subspecies other than subspecies *enterica* that more commonly occur in poikilothermic animals and in the natural environment[405] and from the presence of key virulence factors in exotic serovars.[203] Domestic reptilian pets are well documented as reservoirs of exotic serovars and as sources of salmonellosis in children.[2,174,584,702] Evidence that the disease can degenerate readily into chronic and debilitating clinical conditions condemns any complacency in maintaining the highest level of sanitation in homes and in health care facilities.

Although *S.* Typhimurium has consistently dominated national reports of human salmonellosis prior to the mid-1980s,[160] the ongoing pandemic of *S.* Enteritidis, attributed primarily to the transovarian transmission of this invasive serovar in commercially reared chickens, has dramatically altered the statistics of human *Salmonella* infections in recent years. *S.* Enteritidis is now the most common cause of human salmonellosis in many European countries—notably England and Wales,[206] Spain,[665] Scotland,[600] Italy,[592] and France.[291] Factors that may contribute to the introduction of new serovars include importation of contaminated foods and food ingredients from countries with inadequate hygienic controls, return of travelers infected abroad with exotic *Salmonella* serovars, selection of resistant serovars due to the widespread use and abuse of antibiotics in human medicine and in agricultural and aquacultural food production, seasonal migration of birds and other wildlife, and the unrestricted movement of contaminated surface waters. The following episodes exemplify the important role of international food trade in the dissemination of uncommon *Salmonella* serovars. Cocoa beans imported from Africa were incriminated in a major chocolate outbreak of *S.* Eastbourne in Canada and the US. This serovar, which rarely had been encountered in either country previously, virtually disappeared after the outbreak.[146,164,177] An outbreak in England and Wales associated with importation of Italian chocolate contaminated with *S.* Napoli also subsided without entrenchment of the bacterium in the environment or in the food chain.[495] The inability of uncommon serovars to establish themselves in human populations and/or the food chain may result from the effective containment and treatment of infected human cases, thereby preventing further dissemination of the bacterium, or from its competitive exclusion by natural endogenous microflora in potentially suitable ecological niches.

45.3.4 Antibiotic Resistance

The increasing occurrence of antibiotic-resistant *Salmonella* spp. has compromised the therapeutic efficacy of first-line antimicrobial drugs such as ampicillin, chloramphenicol, and trimethoprim sulfamethoxazole for the treatment of typhoid and paratyphoid fever.[296,515,568,651] Failure to effectively deal with this growing human health threat could result in the widespread contamination of the global food supply with multiple antibiotic-resistant salmonellae and other bacterial pathogens. The public health concern for the growing prominence of resistant *Salmonella* is heightened by the propensity for the conjugative transfer of resistance (R) plasmids.[166] Linked resistance whereby a single R-plasmid encodes resistance to multiple antibiotics is a problem in both human and veterinary medicine. In recent years, treatment in the UK of a septicemic patient infected with *S.* Typhimurium DT 104 with ampicillin could prove unsuccessful and select for strains that carry a 60 megadaltons plasmid-encoded multiple resistance to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamides (Su), and tetracycline (T). The R-type ACSSuT of *S.* Typhimurium, which was first recognized in the UK in 1984, is progressively acquiring resistance to trimethoprim and ciprofloxacin.[677] This phenotype is now the second most common *Salmonella* isolated from humans in England and Wales, overshadowed only by *S.* Enteritidis PT4. The prevalence of R-type ACSSuT in the US also has increased sharply in recent years and accounted for 32% of all human *S.* Typhimurium isolates in 1996.[37]

The high resistance of *S.* Typhimurium to A, C, trimethoprim and SXT in most of the listed countries contrasts sharply with the generally lower level of resistance encountered with other serovars (Table 45–3). The acquisition of such resistance probably resulted from the continued prominence of *S.* Typhimurium in human disease and the widespread use of A, C, and SXT in the treatment of bacterial infections. It will be interesting to follow whether the gradual abandonment of A, C, and SXT in human medicine in favor of newer drugs such as third-generation cephalosporins and fluoroquinolones will result in a marked reduction in the resistance of *Salmonella* to A, C, and SXT. The recent introduction of ciprofloxacin and other fluoroquinolones for the treatment of systemic salmonellosis and the chronic *Salmonella* carrier state may have fostered the emergence of ciprofloxacin-resistant *S.* Typhimurium and *S.* Enteritidis in England and Wales and in the US, respectively. The increased ciprofloxacin resistance of *S.* Hadar from 2.0% to 39.6% between 1991 and 1994 in England warrants close monitoring.[247] Ofloxacin resistances of ≤7.0% in human *Salmonella* isolates were reported in France,[98] Hong Kong,[375] and India,[466,682] whereas 56.8% of 73 strains of *S.* Typhimurium in Brazil were resistant to pefloxacin.[45] Nalidixic acid resistance was reported in 93.0% of human *S.* Typhimurium isolates in India[682] and in 100% of *S.* Hadar isolates in Belgium.[518] Resistance of *Salmonella* spp. to two or more antibiotics is not uncommon, and several human *Salmonella* isolates in Korea were shown to be resistant to 10 antibiotics.[120] National 2- to 25-fold increases in the number of human *Salmonella* isolates resistant to A and C in recent years cast a bleak prospect for the future (Table 45–4). Less dramatic changes in resistance were reported for trimethoprim (Tp) and SXT in most countries, whereas increases in multiple resistance were generally modest except for Spain where a 31-fold increase was reported for the period 1986 to 1991. From 1981 to 1990, the resistance of bovine isolates of *S.* Typhimurium to A, C, Tp, and multiple antibiotics increased significantly in England and Wales.

The current situation has developed partly from the use and abuse of antibiotics in human medicine and from the continuing widespread use of medically important antibacterials as growth-promoting or prophylactic agents in the agriculture and aquaculture (Section 45.5.2).

45.4 MECHANISM OF PATHOGENICITY

45.4.1 Dynamics of Invasion

Nonspecific host defenses play an important role in averting bacterial infection from the consumption of *Salmonella*-contaminated foods. These constitutive protective mechanisms include antibacterial lactoperoxidase in the human saliva, gastric acidity, intestinal mucoid secretions, peristalsis, and sloughing of enterocytes that synergistically oppose the *Salmonella* colonization of the intestinal mucosa. These natural hurdles are reinforced immunologically by the bactericidal action of epitheliolymphoid tissues (Peyer's patches), phagocytic leucocytes such as monocytes, macrophages, and polymorphonuclear leucocytes and by the lytic activity of host complement and T-lymphocytes.[166] Prerequisites for a successful human *Salmonella* infection include the attachment (colonization) to mucosal enterocytes or to the specialized M cells in Peyer's patches of the small bowel. The subsequent penetration of salmonellae into the cytoplasm of epithelial cells triggers an influx of leucocytes to the infected area and a leucocytic release of prostaglandin resulting in mucosal inflammation. The bacterial release of enterotoxin within the inflamed tissues activates host adenyl cyclase resulting in a diarrheagenic fluid secretion into the intestinal lumen.

Determinants for the invasion phenotype of *Salmonella* are encoded by no fewer than 28 genes located in a contiguous 35 to 40 kb region at centisome 63 (pathogenicity island) of the *Salmonella* chromosome. The low G + C content of this region compared with that found in the remaining chromosomal DNA suggests that the entry genes were acquired in block by horizontal transfer from another microorganism. The invasion region of the chromosome corresponds to a contact-dependent type III secretion system for the delivery of bacterial proteins involved in the invasion of targeted eukaryotic cells.[248] The loci for invasion (*inv*), the surface presentation of antigen (*spa*), the *Salmonella* invasion protein (*sip*), the *pho P* repressed gene (*prg*), the hyperinvasive locus (*hil*), and the oxygen-regulated gene (*org*) figure prominently in this protein export system. Ambient osmolarity and oxygen tension modulate the bacterial expression of the *inv* locus by

Table 45–3 Prevalence of Antibiotic Resistance in Human Isolates of *Salmonella*

				Antibiotic Resistance[a] (% of Strains)							
Country	*Period*	*Number of Strains Tested*	*Serovar*	*A*	*C*	*Tp[b]/SXT[c]*	*Su*	*G*	*Cip*	*Multiple[d]*	*Reference*
Bangladesh	1990–1992	236	S. Typhi	66.5	78.8	72.9[c]	NL[e]	0	0	NL	574
Brazil	1987–1992	116	multiple	73.3	62.9	50.0[c]	NL	64.5	NL	73.3	45
England and Wales	1995	3,837	S. Typhimurium	≥87.2	≥87.2	≥33.1[b]	≥87.2	NL	6.2	≥87.2	648
France	1994	82	S. Typhimurium	100.0	78.0	1.2[c]	95.0	0	NL	95.8	115
	1994	2,558	multiple	22.7	18.5	8.6[c]	NL	0.2	NL	NL	98
Greece	1991–1993	377	multiple	36.1	1.8	5.3[c]	NL	1.1	NL	NL	393
Hong Kong	1983–1993	68	S. Enteritidis	4.4	0	1.5[c]	NL	0	NL	NL	375
		517	S. Typhimurium	60.9	62.6	38.5[c]	NL	34.2	NL	NL	
India	1988–1989	29	multiple	93.1	100.0	NL	NL	10.3	NL	NL	664
	1989–1990	190	S. Typhimurium	82.6	80.0	75.3[b]	86.8	0	0	87.9	466
	1991	85	S. Typhimurium	93.0	94.0	96.0[c]	NL	NL	NL	NL	682
Japan	1988–1990	47	S. Hadar	64.9	0	NL	NL	NL	NL	85.1	465
Jordan	1978–1989	≤ 495	S. Typhimurium	90.0	84.0	79.0[c]	90.0	8.0	NL	NL	604
Kenya	1990–1992	100	S. Typhimurium	45.0	16.0	39.0[c]	NL	5	0	NL	378
Saudi Arabia	1989–1991	54	multiple	30.8	32.0	37.5[c]	NL	0	NL	NL	438
	1989–1991	46	S. Enteritidis	13.6	16.7	NL	NL	0	NL	NL	
	ca 1996	153	multiple	16.0	11.0	11.0[c]	NL	0.7	0	16.0	376
Spain	1991	274	multiple	30.3	15.7	2.6[c]	NL	NL	NL	15.0	471
Taiwan	1987	121	S. Typhimurium	95.0	92.0	51.0[c]	NL	39.0	NL	NL	504
Tunisia	1980–1991	177	S. Wien	100.0	94.0	27.0[c]	NL	60.0	NL	NL	69
United States	1989–1990	758	multiple	13.0	4.0	1.0[c]	15.0	5.0	0	25.0	410
	1990–1992	127	S. Enteritidis	37.0	1.0	NL	0	1.0	2.0	35.0	475
Vietnam	1992–1993	39	S. Typhi	69.2	76.3	71.8[c]	76.9	NL	NL	NL	12

[a] Ampicillin (A); chloramphenicol (C); sulfonamides (Su); gentamicin (G); ciprofloxacin (Cip)

[b] Trimethoprim

[c] Trimethoprim sulfamethoxazole

[d] Resistant to ≥ 2 antibiotics that may not be listed above

[e] Not listed

Table 45–4 Temporal Changes in the Antibiotic Resistance of *Salmonella*

				Antibiotic Resistance (%)				
Country	*Period*	*Serovar*	*Number of Strains Tested*	*A*[a]	*C*[a]	*Tp*[b]*/SXT*[c]	*Multiple*[d]	*Reference*
HUMAN								
Belgium	1991	S. Hadar	67	16.4	NL[e]	NL	NL	518
	1994		464	79.7	NL	NL	NL	
England and Wales	1981	S. Typhimurium	3,932	5.0	5.0	8.0[b]	5.0	647
	1990		5,451	17.0	6.0	21.0[b]	19.0	
Hong Kong	1985	multiple	89	6.7	13.5	1.1[c]	NL	424
	1988		299	27.1	34.4	8.4[c]	NL	
Spain	1980–1982	S. Typhimurium	66	15.2	7.2	<1.0[c]	10.6	544
	1992–1994		30	73.3	46.7	6.0[c]	60.0	
Spain	1986	multiple	322	12.1	2.8	1.2[c]	0.6	552
	1991		300	34.0	15.3	0.6[c]	18.6	
Turkey	1982–1987	S. Typhi	16	0	0	0	NL	595
	1988–1992		11	23.5	17.6	17.1[c]	NL	
United Kingdom	1978–1985	S. Typhi	2,345	0.1	0.3	0[b]	NL	651
	1986–1989		790	1.0	1.5	0[b]		
	1990		248	21.0	20.0	22.0[b]		
	1993		194	25.0	25.0	25.0[b]		
United States	1979–1980	multiple	378	9.5	1.1	0[c]	17.0	410
	1984–1985		341	10.3	2.6	0.6[c]	26.0	
	1989–1990		484	14.5	2.9	0.4[c]	31.0	
NONHUMAN								
England and Wales	1981	S. Typhimurium	1,157[f]	13.0	15.0	8.0[b]	15.0	647
	1990		1,178[f]	66.0	45.0	53.0[b]	66.0	
	1981	S. Typhimurium	49[g]	22.0	22.0	35.0[b]	22.0	
	1990		144[g]	19.0	3.0	35.0[b]	35.0	
	1981	S. Typhimurium	117[h]	<1.0	<1.0	0[b]	1.0	
	1990		1,187[h]	6.0	2.0	7.0[b]	8.0	

[a] Ampicillin (A); chloramphenicol (C)
[b] Trimethoprim
[c] Trimethoprim sulfamethoxazole
[d] Resistant to ≥ 2 antibiotics that may not be listed above
[e] Not listed
[f] Bovine
[g] Porcine
[h] Poultry

altering the helicity of chromosomal DNA and the transcription of genes within the pathogenicity island.[250] Surface contact between invasive salmonellae and the microvilli of enterocytes engenders the bacterial formation of transient proteinaceous appendages that are deemed essential for *Salmonella* invasion.[275] These appendages, termed invasomes, are much thicker than flagella and type-1 fimbriae and measure 0.3 to 1.0 μm in length and approximately 60 nm in diameter.[250] The infrastructure and role of invasomes remain elusive.[249] Several *inv* loci are linked functionally in the energy-dependent assembly of these surface organelles.[136] A synthetase enzyme encoded by the *inv I* locus together with an uncharacterized gene product from the *inv J* locus provide the building blocks for the bacterial appendages, which are exported to the cell exterior by an *inv G*-encoded translocase driven by an *inv C*-encoded ATPase.[166]

The next event in *Salmonella* invasion involves bacterial signals that induce the polymerization of actin and cytoskeleton rearrangement within the enterocyte cytoplasm and the formation of membrane ruffles or blebs on the surface of intestinal epithelial cells and of M cells in Peyer's patches. Ultimately, the membrane ruffles engulf and draw the adherent *Salmonella* cells into the enterocyte cytoplasm by macropinocytosis.[246] These Ca^{+2}-dependent events are elicited by signal transduction from the invading microorganism where the high enterocyte Ca^{+2} concentration required for actin polymerization derives from two independent mechanisms. The release of bacterial gene products encoded by the genomic *inv E* locus produces a net influx of lumenal Ca^{+2} into the host cell cytoplasm.[276] The signal transduction for Ca^{+2}-dependent entry into host cells is also linked to the *Salmonella*-induced phosphorylation of tyrosine in host mitogenic activating protein kinase and in the epidermal growth factor receptor.[249,566] The identity of the *Salmonella* inducer is uncertain. The second mechanism that contributes to the high Ca^{+2} concentration in enterocytes relates to a *Salmonella* induction of host phospholipase C activity, resulting in the breakdown of enterocyte membrane lipids and intracytoplasmic release of inositol triphosphate that liberates cytoplasmically bound Ca^{+2}. The appearance of host cell ruffles coincides with the disappearance of the surface appendages on adherent salmonellae. The *Salmonella* invasion gene (*sip*) encodes the important Sip B, C, and D secretory proteins for bacterial entry into host cells. Although their mechanisms of action have yet to be confirmed, formation of transmembrane channels in the mammalian cell may be involved.[249]

Salmonellae are internalized within pinocytotic vacuoles whose membranes predominantly contain MHC class 1 proteins.[246] These MHC proteins originating from a *Salmonella*-directed aggregation of enterocyte membrane proteins putatively conceal the presence of an infected vacuole from immunological host defenses.[255] The reversion of actin microfilaments and membrane ruffles to their basal states coincides with the formation of endocytic vacuoles. Further maturation of the infected vacuole results in a decrease in membrane MHC 1 proteins in favor of lysosomal glycoproteins, which may protect against the degradative action of host cell enzymes.[254] In contrast to *Yersinia*, *Shigella*, and enteroinvasive *E. coli* that replicate within the cytoplasm of host cells, *Salmonella* multiply within vacuoles.[253] The concurrent appearance of filamentous structures containing acid phosphatase and lysosomal membrane glycoproteins that seemingly link the infected vacuole to the epithelial cytosol was recently described. The formation of these organelles of unknown function requires viable salmonellae within endocytic vacuoles.[256] The infected vacuoles translocate from the apical pole to the basal membrane of the host cell where salmonellae are released into the lamina propria. The binding of host plasminogen and its specific activator to *Salmonella* fimbriae and subsequent conversion to proteolytic plasmin may enable salmonellae to permeate host tissue barriers and migrate into deeper host tissues.[610]

45.4.2 Virulence Factors

In addition to the genomic *inv*, *sip*, *hil*, and *prg* loci that facilitate bacterial invasion of host cells, other virulence factors potentiate the survival and/or growth of *Salmonella* in the hostile mammalian host environment. The expression of many genes encoding virulence determinants is triggered by a variety of host-dependent signals.

Iron is essential for the maintenance of mammalian cells and for the growth and survival of salmonellae.[148] The finite amount of cationic iron available in mammalian tissues leads to strong competition between both cellular antagonists. The host transferrin- and lactoferrin-dependent sequestering of iron and subsequent intracellular storage of captured iron as ferritin-iron (III) complexes potentially is bacteriostatic. *Salmonella* rely on two iron-scavenging siderophores chromosomally regulated by the *fur* gene to satisfy their requirement for this cation.[166] The high affinity of the phenolate siderophore termed enterochelin or enterobactin enables it to dislodge and sequester ferric ions previously bound to mammalian iron-binding proteins.[436] A lower affinity hydroxamate aerobactin also competes with host chelators for ferric cations. The bacterial uptake of ferric-siderophore complexes is mediated by an outer membrane protein (OMP) receptor previously induced by iron depletion in the bacterial cell. The cytoplasmic internalization of the ferric-siderophore complex coincides with a reduction in the oxidative state of the internalized complex. Consequently, ferrous ions are released into the bacterial cytoplasm because of the low siderophore affinity for iron (II) cations. The decreased virulence of salmonellae under conditions of iron deprivation underlines the importance of bacterial siderophores in pathogenesis.[436]

Salmonellae are responsive to marked changes in the osmolarity, temperature, oxygen tension, oxidative stress, and low nutrient levels in their microenvironment. The *omp R* and *env Z* genomic loci constitute a two-component system that regulates the synthesis of the Omp C and Omp F transmembrane porins in response to prevailing osmolar conditions. The Omp R transcriptional regulator is activated by signal transduction from the inner membrane Env Z sensor. The importance of this system in pathogenicity is evidenced by the attenuation of strains carrying a mutation in *omp R* or a double mutation in the *omp C* and *omp F* loci. A mutation in either porin locus does not affect the virulence of carrier strains.[209] The rapid synthesis of heat-shock proteins in prokaryotic and eukaryotic cells is induced by stress conditions such as major upshifts in temperature, extreme pH values, and presence of toxic products from the oxidative burst of phagocytes. These proteins function as molecular chaperones or as proteases.[292] Chaperones ensure that newly synthesized proteins are assembled into an active configuration, whereas proteases degrade abnormal or dysfunctional peptides. The highly discriminating and corrective functions of heat-shock proteins attest to their importance in the survival of *Salmonella* during its infectious cycle. The oxygen tension in the microenvironment can also induce the expression of invasion genes. Low levels of oxygen, presumably similar to that found in the intestinal lumen, increased the invasiveness of *S.* Typhimurium into cultured mammalian cells, whereas growth under high oxygen tension markedly reduced invasiveness.[436] The systemic migration of salmonellae in the host exposes the microorganism to phagocytosis by macrophages, monocytes, and polymorphonuclear leukocytes and to the antibacterial conditions within these host defense cells.[162,166] The toxicity of singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals generated during the oxidative burst of phagocytes is minimized through the degradative actions of bacterial superoxide dismutase, peroxidase, and catalase enzymes and by the cytotoxic product-dependent induction of the *oxy R* locus that regulates the synthesis of protective proteins.[166,522] The resistance of *oxy R* mutants of *S.* Typhimurium to phagocyte inactivation does not support the protective role putatively ascribed to the *oxy R*-dependent proteins.[501] The chromosomal *rpo S* locus is induced under conditions of nutrient deprivation as encountered in the infected phagosomes of host macrophages or in salmonellae entering the stationary phase of growth. The alternative sigma factor (σ^s) encoded by the *rpo S* gene couples with core RNA polymerase to form the holoenzyme.[402] The proper alignment of the polymerase holoenzyme with its targeted gene encoding a protective protein derives from the σ^s-dependent recognition of a specific promoter sequence in the targeted gene. The σ^s factor thus identifies the DNA domain where gene transcription begins. The transcription of numerous genes and operons is under σ^s control.[427]

An important facet of acid resistance in *Salmonella* relates to the genomic *phoP/phoQ* regulon that facilitates the survival of salmonellae within the acidic environment of phagocytes. This two-component transcriptional regulator system also protects against the lytic action of nonspecific cationic peptides (defensins) produced by phagocytes and epithelial cells.[166,460,673] The gene product of the *pho Q* locus is a kinase that senses unfavorable acidic conditions in the cell exterior by means of a short amino acid sidechain that extends into the periplasmic space of the bacterial cell envelope. The sensorial attributes of Pho Q are due, apparently, to acidic amino acid residues in the sidechain (anionic box) and their putative ability to recognize cationic defensins and the high proton concentration in the phagocyte cytoplasm. The activated kinase then relays the environmental signal through autophosphorylation and a kinase-dependent transfer of phosphate to the *phoP* transcriptional activator protein.[460] The phosphorylated PhoP subsequently activates the transcription of 18 genes, including the *pagA* to *pagP*, *psi D*, and *pho N* loci. The gene products of these loci do not confer protection against defensins; however, the *pag C* gene encodes a virulence-determining OMP that protects against the lytic attack of serum complement, whereas the gene products of the *pag D, pag J, pag K,* and *pag M* loci enhance *Salmonella* survival in macrophages.[65] In contrast to *phoP*-activated gene (*pag*) loci, which are induced in response to unfavorable environmental conditions, *phoP*-repressed genes (*prg*) are expressed under nonstress conditions and collectively encode about 20 proteins involved in bacterial invasion of epithelial cells, increased pinocytosis, membrane ruffling, or formation of spacious phagosomes in macrophages.[64,166]

Virulence plasmids play an important role in the pathogenicity of *Salmonella* spp. These cytoplasmic DNA organelles occur in low copy numbers and replicate independently of the bacterial chromosome. They occur in a limited number of serovars and, surprisingly, are absent in typhoid and paratyphoid strains.[162,293] *Salmonella* virulence plasmids vary from 30 to 60 MDa in size and contain highly conserved nucleotide sequences (structural homology) capable of restoring the virulence of plasmid-cured strains (functional homology). However, the conjugative transfer of plasmids between *Salmonella* strains occurs infrequently as suggested by the remarkable similarity of virulence plasmids within a given serovar and marked differences between plasmids of different serovars.[294] Virulence plasmids encode factors that promote the systemic spread of salmonellae in host tissues and the bacterial proliferation in macrophages.[295] A highly conserved 6.4 to 8kb region figures prominently in *Salmonella* virulence plasmids.[629] The transcription of this genetic locus, termed *Salmonella* virulence plasmid (*spv*), is induced on bacterial exposure to acidic conditions within phagocytes and other microenvironments, elevated temperatures, and nutrient deprivation.[634] The *spv* operon consists of five open reading frames corresponding to a regulator gene (*spv R*) and a cluster of four structural genes (*spv ABCD*). One of the two promoter regions within the *spv* operon lies upstream from *spv R*, whereas the second promoter is positioned between the *spv R* and *spv A* loci.[295] Transcription of the *spv ABCD* gene cluster progresses sequentially from left to right under the combined control of the *spv R* regulator protein and the alternate sigma factor (σ^s) encoded by the *rpo S* locus.[123,394] The yield of gene products from the transcription of the *spv A* to *spv D* loci occurs in decreasing amounts and is greater in stationary than in log phase cells.[294] The σ^s is the principal activator of the *spv R* locus through its interaction with the *spv R* promoter region, and overshadows the minor involvement of Spv R as a self-regulator of the *spv R* locus.[123,310] The role of the *spv ABCD* gene products in conferring strain virulence is poorly understood. Transposon insertion experiments with *S.* Dublin recently demonstrated that *spv R* and *spv B* are essential virulence genes, whereas *spv C* and *spv D* played a secondary but necessary role for the development of full strain virulence. The *spv A* locus reportedly did not contribute to strain virulence.[294] In contrast, a 10-fold greater expression of *spv A* in *S.* Dublin than in *S.* Typhimurium was tentatively associated with the high virulence of *S.* Dublin.[634] Other genetic determinants that enhance bacterial pathogenicity also occur on *Salmonella* virulence plasmids.[293] These include the resistance to complement killing (*rck*) locus whose gene product blocks the formation of a functional C5b-9 complement membrane attack complex and prevents its lytic insertion into the bacterial outer membrane. The plasmidic replication (*rep*) and stability (*par, rsd, crs*) loci that ensure stable plasmid maintenance are not uniformly distributed among serovars that carry virulence plasmids.

Several bacterial surface structures contribute to the virulence of *Salmonella* spp. A polysaccharide capsular antigen designated Vi occurs in *S.* Typhi and *S.* Paratyphi C but rarely in *S.* Dublin.[522] The Vi capsule inhibits the binding of the C3b host complement factor to the bacterial LPS, a prerequisite for the complement lysis of salmonellae and induction of macrophage phagocytosis.[162] The length of LPS on the bacterial surface also affects complement lysis. In smooth variants, the long LPS sterically hinders the stable insertion of the C5b-9 lytic complex into the outer membrane. Rough strains with a short LPS lack this protective attribute. It follows that smooth variants exhibit greater virulence than rough *Salmonella* strains. Therefore, the Vi capsular antigen, the plasmidic *rck* locus, and the surface LPS play an important role in bacterial resistance to host complement lysis. The previously discussed Omp C and Omp F outer membrane porins are equally important surface structures because they potentiate greater tolerance to wide osmolar fluctuations in the bacterial microenvironment (Section 45.4.1).

Several toxins, including endotoxin, enterotoxin, cytotoxin, *zot* (zonula occludens toxin)-like protein, and salmolysin, are associated with *Salmonella* spp.[660] The endotoxin consists of three molecular domains: an outer hexose polysaccharide chain that provides for the serotypic differentiation of strains, a core polysaccharide chain consisting of hexose (outer) and heptose (inner) units, and an innermost lipid A moiety that triggers the endotoxic response in infected hosts. The cryptic positioning of lipid A within the bacterial outer membrane hampers the induction of host inflammatory reactions pending the lytic release of lipid A into host tissues. On release, lipid A activates the leucocytic production of cytokines that trigger the inflammatory response. Endotoxins released in host cells also can induce the expression of host surface receptors that can serve as enterotoxin binding sites. Most strains of *Salmonella enterica*, but none belonging to *Salmonella bongori*, possess the enterotoxin gene (*stn*). This locus is located on a 2.8 kb DNA segment that maps at 89.7 minutes on the chromosome and encodes a 29 kDa thermolabile protein. *Salmonella* enterotoxin induces the elongation of Chinese hamster ovary (CHO) cells but fails to alter the morphological integrity of Y-1 adrenal and HeLa cell monolayers. The cytotonic enterotoxin does not share any nucleic or amino acid homology with cholera toxin nor with the thermolabile enterotoxin (LT) of *Escherichia coli.*[660] The ability of cholera antitoxin to neutralize *Salmonella* enterotoxin remains controversial.[126,541,660] Serological and electrophoretic pattern studies suggest that the *Salmonella* enterotoxin consists of A (active) and B (binding) subunits. The A moiety activates host cell adenylate cyclase, whereas the B subunit binds to the GM_1 ganglioside of intestinal enterocytes.[121,126,166,237] The instability of enterotoxin at $pH < 6.0$ points to its limited role in *Salmonella* pathogenicity during the inclusion of the pathogen in phagolysosomes and acidified epithelial vacuoles. *Salmonella* spp. also produce a thermolabile cytotoxin associated with the outer membrane.[47,439,554] The trypsin- and protease-sensitive cytotoxin is stable between pH 7.0 and 8.0 but is inactivated rapidly outside this pH range.[200,439] The cytotoxin is not related serologically or genetically to Shiga-like toxins as evidenced by the inability of Shiga and *E. coli* Vero 1 and 2 antitoxins to neutralize *Salmonella* cytotoxin, and by the failure of Shiga toxin-specific probes to hybridize with total *Salmonella* DNA.[47,200] Maximal production of cytotoxin occurs in the early stationary phase of growth at pH 7.0 and diminishes as the incubation temperature increases from 35° C to 42° C or as the pH shifts from neutrality.[200] Although the cytotoxic action mechanism is poorly understood, inhibition of protein synthesis in host cells[390] and membrane disruption through a cytotoxin-dependent chelation of Ca^{+2} and Mg^{+2} associated with host cell membranes have been reported.[508] Clearly, these lytic events enhance strain virulence by facilitating the dissemination of salmonellae within host tissues. A similar function has been ascribed to a 50 kDa Zot-like protein encoded on a 5.5 kbp genomic DNA segment that maps between 10 and 20 min on the chromosome and occurs infrequently in *Salmonella* strains.[660] The toxin disrupts the intercellular tight junction of enterocytes, thereby facilitating the migration of salmonellae from an infected enterocyte into a neighboring host cell. Synergism between the destructive effect of cytotoxin and Zot proteins on host cells, the endotoxin-induced inflammatory response, and the enterotoxin-dependent efflux of water and electrolyte in enterocytes all contribute to the clinical presentation of human salmonellosis. Finally, a poorly characterized hemolytic salmolysin is also associated with *Salmonella* spp. This thermolabile 16.7 kDa protein is encoded by the *sly A* locus. The observation that the median lethal dose of a *sly A* mutant of *S.* Typhimurium in mice was markedly greater than that for the wild-type parent indicates a role of salmolysin as a virulence determinant.[417]

45.5 INCIDENCE OF THE ORGANISM IN THE ENVIRONMENT AND IN FOODS

45.5.1 Agriculture and Aquaculture

The ubiquity of *Salmonella* spp. in the natural environment contributes significantly to the continued prominence of salmonellae in meat animals and derived products (Table 45–5). The on-farm infection of cattle follows from a liberal access to surface waters contaminated with industrial and farm effluents or from infected wildlife. The potentially high organic content of surface waters together with climatic conditions that favor the growth of bacteria could expose farm animals to massive doses of salmonellae. Spread of *Salmonella* also could stem from the repeated contact of animals with carriers within the herd or from the consumption of contaminated commercial feeds. Feeding of untreated animal wastes to livestock also contributes to herd infection.[298] Intensive husbandry in the porcine and poultry industries as well as animal behavior creates difficulties in maintaining adequate hygienic conditions in rearing pens, and in limiting cross-contamination between animals. The introduction of infected poultry replacement stocks in sanitized premises, unrestricted access of birds to fecally contaminated communal drinking stations, provision of *Salmonella*-contaminated poultry feed, and the high density of birds in rearing facilities synergistically

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contribute to the continued prominence of salmonellae in poultry operations. Animal feeds likely are a major reservoir of *Salmonella* spp. (Table 45–6). Although the large-scale thermal processing of animal offals targets the destruction of pathogenic bacteria, the operation of cookers at suboptimal conditions, contamination of rendered feeds with raw product, and the bulk storage and shipment of rendered animal proteins can undermine the quality and safety of finished products.[71] Raw materials of vegetal origin also can introduce salmonellae in finished feed products (Table 45–6).[20,671] Although the numbers of *Salmonella* in feed and feed ingredients tend to be low, values of 10^3 salmonellae $(100\ g)^{-1}$ of product have been reported.[71,75,169,180] The efficacy of pelleting in markedly reducing the bacterial load in feed mash relies on the processing of materials at ≥ 80° C and an adjusted 15% moisture content.[319,450,671] The value of propionic, formic, lactic, fumaric, and citric acids in the postprocess decontamination of feeds has been examined.[73,320] The efficacy of commercial mixtures of propionic and formic acids (BioAdd™, Sal Curb™) in reducing the vertical transmission of *Salmonella* in animal husbandry has yet to be confirmed. There is evidence, however, that these feed additives may also exert an antibacterial effect in the crops of birds, thereby affording protection against other environmental sources of infection.[320,346]

Slaughtering-plant operations generally amplify the level of bacterial contamination associated with live animals. The stress-induced shedding of *Salmonella* during transportation from the farm to the slaughterhouse and the containment of animals in holding pens before slaughter markedly increase the propensity for cross-contamination.[93,468,469,477,706] The hygienic removal of fecally soiled animal hides and the risk of cross-contamination of carcasses during the cutting, trimming, and rinsing of animal tissues pose a major challenge to the meat industry.[93,272,469,612] The situation is particularly difficult in poultry slaughterhouses that receive stressed birds with fecally contaminated body surfaces. After electrical stunning and bleeding, birds are hung on mechanical lines and briefly passaged through a common immersion scald tank operated at 50° C to 60° C.[490,611] Scalded birds are then defeathered by the shearing force of a rotary drum picker that spreads bacterial surface contamination between carcasses.[133,703] Scalding, defeathering, and evisceration are critical control points in the hygienic processing of poultry. The wide variations in the reported national prevalence of salmonellae in preslaughter and postslaughter animals (Table 45–5) likely reflect differences in the endemicity of the microorganism in individual countries, the nature of test materials, the stringency of sampling, and the analytical method used to detect the organism. The reported national incidence of salmonellae in retail meats varies widely (Table 45–7). Inadequate refrigeration and hygienic control during slaughtering and distribution, and the mishandling of meat products in local butcher shops, undoubtedly contribute to the generally higher level of contamination in meat products from developing countries. The high prevalence of *Salmonella* in retail poultry reflects the continued inability of the industry to effectively prevent the spread of this microorganism during the production, slaughtering, and marketing of raw poultry meat (Table 45–8).

Horizontal spread of salmonellae among layer hens and vertical transmission of the bacteria on the shell egg surface and within the egg are problems for the egg industry (Table 45–9). Although the aqueous sanitizing of shell eggs at 41° C and pH ≥10.0 reduces external surface contamination, the practice disrupts the structural integrity of the outer protective cuticle and facilitates penetration of salmonellae through exposed pores in the egg shell.[329,449,477] Concerns on the safety of intact shell eggs have increased dramatically in recent years because of the possible transovarian transmission of *S.* Enteritidis into the egg magma prior to shell deposition.[144,561] The transovarian infection of eggs also has been associated with other serovars including *S.* Thompson, *S.* Menston, *S.* Typhimurium, *S.* Pullorum, and *S.* Gallinarum.[60,282] Internally contaminated shell eggs pose a significant public health risk unless the eggs are boiled for more than 8 min or cooked until the yolk is well congealed.[343] The prevalence of internally contaminated shell eggs generally is very low (Table 45–9).

Salmonellae are often present in ethnic raw meats (Table 45–10) and in raw fish and shellfish (Table 45–11). Although marine waters generally are considered to be free of *Salmonella* spp., estuaries and coastal waters contaminated with human and agricultural wastes pose a human health hazard (Table 45–11). The use of rinse water and packaging ice from contaminated sources, poor personal hygiene among nonspecialized workers, and repeated manual handling of raw products during the harvesting and packaging operations may have contributed to the higher rates of contamination in fish and shellfish from Asia. The rapid depletion of feral stocks of marine and freshwater species coincident with a growing consumer demand for these products favored an acceler-

Table 45–5 Reported Incidence of *Salmonella* in Meat Animals

	Number of Samples		
Product	*Tested*	*Percent Positive*	*Reference*
ON FARMS (herds, flocks)			
Beef/cattle			
Indonesia (1986)	77	3.9	476
Malta (1993)	300	41.3	672
Pork			
Denmark (1995)	15,797	5.5	34
Indonesia (1989)	236	11.0	476
United States (ca 1995)	140	35.7	32
Chicken (broilers)			
Denmark (1995)	4,168	24.0	34
Japan (ca 1990)	286	24.1	655
Japan (1989)	340	5.0	465
Switzerland (1991)	92	16.6	62
The Netherlands (1992–1993)	181	27.0	355
Turkey			
Denmark (1995)	173	18.5	34
Buffalo			
Indonesia (1986)	14	35.7	476
POSTSLAUGHTER			
Beef			
Denmark (1995)	2,165	0.6	34
Germany (1991)	18,242	5.1	304
Nigeria (ca 1989)	118	4.2	3
Portugal (1987–1988)	408	20.3	79
United States (1993)	2,112	2.7	33
Pork			
Australia (ca 1995)	1,428	13.1	32
Canada (1985–1986)	448	10.0	434
Denmark (1995)	18,032	0.81	34
Germany (1991)	34,137	1.7	304
India (ca 1988)	480	3.5	609
India (ca 1989)	114	17.5	707
Japan (1984–1989)	1,717	5.7	710
Japan (1992)	69	5.8	352
Portugal (1987–1988)	405	5.4	79
Romania (1989–1990)	260	13.1	498
United States (ca 1995)	450	1.7	575

continues

Table 45–5 continued

Product	*Number of Samples*		*Reference*
	Tested	*Percent Positive*	
Chicken			
Cuba (ca 1990)	200	62.5	208
Denmark (1994)	8,300	36.4	25
Denmark (1995)	4,099	45.7	34
Italy (ca 1990)	172	10.5	119
Japan (ca 1990)	286	24.1	655
Japan (1992)	238	33.6	352
Portugal (1986–87)	300	55.0	78
Thailand (1991–92)	353	51.0	360
United States (1990–91)	200	34.9	99
(1994–95)	1,297	20.0	31
Buffalo			
India (ca 1988)	78	5.1	52
Goats			
India (ca 1989)	50	6.0	542
Sheep/lamb			
Germany (1987)	264	9.8	361
India (ca 1989)	61	1.6	542
Iran (ca 1992)	500	2.6	633
Portugal (1987–1988)	300	7.3	79
Spain (ca 1995)	30	10.0	606

Table 45–6 Reported Incidence of *Salmonella* in Animal Feeds

Country of Origin	*Product*	*Number of Samples*		*Reference*
		Tested	*Percent Positive*	
Rendered Animal Protein				
Australia (ca 1995)	Meat meal	72	30.6	32
Lebanon (ca 1988)	Animal feed	300	19.0	474
The Netherlands (1990–1991)	Fish meal	130	31.0	671
United States (ca 1994)	Animal protein	101	56.4	20
Vegetable Protein				
The Netherlands (1990–1991)	Maize grits	15	27.0	671
United States (ca 1994)	Vegetable protein	50	36.0	20
Complete Feed				
Brazil (ca 1995)	Poultry feed	200	10.0	74
Denmark (1995)	Swine feed	2,300	0.7	34
	Animal feed	1,669	1.2	
Japan (1988–1990)	Poultry feed	115	3.5	465
The Netherlands (1990–1991)	Poultry feed	360	10.0	671

Table 45–7 Reported Incidence of *Salmonella* in Retail Meats

	Number of Samples		
Product	*Tested*	*Percent Positive*	*Reference*
Beef (Raw)			
Denmark (1995)	2,559	1.3	34
Japan (ca 1990)	52	1.9	655
Japan (1989–1992)	312	1.3	352
Mexico (1986–1987)	54	20.4	67
Thailand (1986)	130	21.5	545
The Netherlands (1986–1990)	636	3.1	187
Pork (Raw)			
Denmark (1995)	18,032	0.81	34
Italy (1980–1989)	502	1.5	95
Japan (ca 1990)	94	3.2	655
Japan (1988–1990)	135	11.1	465
Japan (1992)	69	5.8	352
Mexico (1989–1990)	61	91.8	227
Mexico (1994)	50	76.0	399
Thailand (1986)	130	21.5	545
Raw Minced Meat			
Mexico (1986–1987)	7[a]	57.1	68
	7[b]	28.6	
The Netherlands (1986–1990)	1,813[a,b]	7.0	187
	847[a]	3.2	
United States (1993–1994)	563[a]	7.5	33
Sausages (Raw)			
Brazil (1990–1991)	17	35.3	553
Iraq (1987)	80	35.0	1
Italy (1980–1989)	2,955	2.4	95
Lebanon (ca 1988)	250	11.2	474
Mexico (ca 1993)	221	40.7	67
Mexico (1994)	50	46.0	399
Nigeria (1982–1983)	103	42.0	492
Scotland (1989)	203	9.8	17
United States (ca 1984)	175	27.0	607
Dry Fermented Sausages			
Italy (1980–1989)	240	0.4	95
The Netherlands (1986–1990)	270	5.9	187
The Netherlands (ca 1987)	145	11.0	303
Smoked Sausages			
The Netherlands (1986–1990)	459	7.2	187

[a] Beef
[b] Pork

ated expansion of the aquacultural industry. Asia is responsible for more than half of the world's aquacultural output.[164] Isolated studies on the *Salmonella* status of aquacultural fish and shellfish demonstrate their potential as vehicles of human salmonellosis (Table 45–11). The popularity of raw fish dishes (sushi) in Asia, North America, and Europe together with the abundance of culinary recipes involving lightly cooked fish and shellfish expose consumers to potentially serious health problems.

Raw milk is the principal reservoir of *Salmonella* spp. in the dairy industry (Table 45–12). Contamina-

Table 45–8 Reported Incidence of *Salmonella* in Retail Poultry

Product	Number of Samples Tested	 Percent Positive	Reference
Chicken (Carcasses/Cut-up)			
Denmark (1995)	492	6.9	34
France (1994)	616	19.8	263
Germany (1994)	630	28.6	263
India (ca 1995)	76	10.5	598
Italy (1988–1989)	118	20.3	5
Japan (1988–1990)	44	63.6	465
Malaysia (ca 1993)	33	39.0	44
Mexico (ca 1993)	70	68.6	116
Northern Ireland (1994)	140	7.0	694
Thailand (1991–1992)	352	81.5	360
The Netherlands (1994)	607	12.4	263
Turkey (ca 1992)	80	31.3	473
United Kingdom (1991–1992)	325	22.8	517
Minced Poultry			
United States (1995)	162[a]	42.0	29
	165[b]	31.7	
Chicken Liver			
Malaysia (ca 1993)	17	35.0	44
Mexico (1986–1987)	18	11.1	68
Thailand (1991–1992)	132	90.2	360
Chicken Gizzards			
Malaysia (ca 1993)	18	44.0	44
Thailand (1991–1992)	45	80.0	360

[a] Chicken
[b] Turkey

tion arises mainly from inadequately sanitized milking equipment and poor disinfection of teat skin prior to milking. Intramammary *Salmonella* infection associated with subclinical mastitis also could lead to the intermittent shedding of salmonellae in raw milk. Temperature fluctuations from the periodic addition of fresh warm milk into on-farm refrigerated storage tanks encourage the growth of salmonellae and undermine the safety of cheese manufactured from raw or thermized (unpasteurized) milk. Prolonged refrigerated storage of raw milk on dairy farms or in industrial silos also would favor the proliferation of psychrotrophic *Salmonella*.[162,363] Following inoculation into fresh milk that had been autoclaved at 100° C for 17 to 20 minutes, growth of *S.* Typhimurium and *S.* Dublin was not detected during storage at 6° C for 6 months. However, pooled strains of *S.* Typhimurium grew slowly at 8° C and 12° C with lag periods of 600 and 8.8 hours and generation times of 83.5 and 8.45 hours respectively.[379] The persistence or growth of *Salmonella* spp. in raw milk and in nonpasteurized dairy products has led to several important outbreaks of human salmonellosis (Section 45.6).

Several factors can contribute to the *Salmonella* contamination of fresh fruits and vegetables (Table 45–13). These include spray or canal irrigation of growing fields with contaminated water, fertilization of farmlands with untreated animal wastes, contamination of crops by infected wildlife, rinsing or cooling of harvested products with *Salmonella*-contaminated water, and multiple manual handling of fruits and vegetables during harvesting, processing, and packaging.[61,335,345] *Salmonella* can multiply on the surfaces of fruits such as tomatoes[685,716] and cut melons[226,280] and on fresh vegetables manually or mechanically moisturized during their retail display at ambient temperature.[96] Clearly, fresh produce to

Table 45–9 Reported Incidence of *Salmonella* in Egg Layer Flocks and Shell Eggs

Country	Number of Samples: Tested	Number of Samples: Percent Positive	Reference
Chicken Flocks			
Canada (ca 1991)	295	52.9 (fecal/eggbelt)	528
Canada (ca 1992)	480	18.1 (necropsy)	528
Denmark (1995)	347	5.5 (cloacal swabs)	34
Germany (1993)	2,112	13.7 (ceca/liver/spleen)	321
Greece (1987–1990)	10	60.0 (fecal/litter/feeds)	53
United States (1991)	406	86.5 (ceca)	215
United States (1994)	76	67.0 (environment)	26
United States (ca 1995)	50	72.0 (environment)	368
Chicken Eggs			
Canada (ca 1995)	16,560	0.06 (content)	526
Denmark (1995)	14,800	0.1 (shell/content)	34
France (ca 1990)	519	2.3 (shell/content)	70
Germany (1980–1989)	9,244	≤ 0.1 (shell/content)	109
Germany (1990)	70	11.4 (content)[a]	106
Hawaii (1989)	106	9.4 (shell)	125
India (ca 1993)	102	4.9 (shell)	384
	102	0.9 (yolk)	
Spain (1985–1987)	372	1.3 (shell/content)[a]	505
	998	0.6 (shell/content)	
Thailand (1991–1992)	744	13.2 (shell)	577
	744	3.9 (content)	
United States (1994)	647,000	0.03 (content)	26
United States (ca 1995)	90	1.1 (shell)	368
Duck Eggs			
Thailand (1992)	564	12.4 (shell)	577
	564	11.0 (content)	

[a] Eggs from sources associated with outbreaks of human salmonellosis.

Table 45–10 Reported Incidence of *Salmonella* in Other Meats

Product	Number of Samples: Tested	Number of Samples: Percent Positive	Reference
Camel			
Egypt (ca 1994)	100	10.0	220
Iran (ca 1992)	113	12.1	633
United Arab Emirates (1987–1991)	205	10.7	687
Crocodile			
Australia (ca 1991)	287	16.0	441
Australia (1991–1992)	72	19.4	557
Zimbabwe (1991)	160	21.3	433
Feral Pig			
Australia (ca 1991)	154	34.4	72
Kangaroo			
Australia (ca 1991)	81	11.1	72

Table 45–11 Reported Incidence of *Salmonella* in Fish and Shellfish Products

Country of Origin	*Product*	*Number of Samples*		*Reference*
		Tested	*Percent Positive*	
Fish				
India (ca 1989)	Frozen fish	161	11.2	353
	Fresh fish	360	4.4	
(ca 1995)	Frozen fish	120	1.7	598
Mexico (1986–1987)	Fish (filets)	20	10.0	68
(1994)	Fresh fish	221	15.8	225
Thailand (1991–1993)	Frozen fish	1,599	0.4	620
Shellfish				
Australia (1981–1982)	Oysters	71	4.2	230
Cuba (1985–1988)	Oysters	1,177	2.0	117
India (ca 1989)	Frozen shrimp	560	8.0	353
	Dried shrimp	25	4.0	
(ca 1990)	Fresh shrimp	30	56.0	478
	Frozen shrimp	16	28.0	
(ca 1995)	Fresh prawns	500	1.0	531
(ca 1995)	Shrimp[a]	NS[b]	37.5	83
Italy (1987–1988)	Mollusks	117	4.3	142
Malaysia (ca 1993)	Prawns	16	25.0	44
	Dried oysters	7	86.0	
	Shrimp paste	19	10.5	
Northern Ireland (1994)	Mollusks	433	8.0	693
Philippines (1988–1990)	Prawns[a]	NS[b]	16.0	550
Thailand (1991–1993)	Frozen shrimp	3,046	0.1	620
	Frozen mollusks	1,451	0.2	

[a] Aquacultural product
[b] Not stated

be consumed raw should always be rinsed thoroughly with potable water. Recent reports on the occurrence of salmonellae in ready-to-eat sesame seed products such as tahineh and halawa have raised new concerns on the safety of these widely distributed ethnic foods (Table 45–13). Both products are derived from roasted sesame seeds dehulled and milled to an oily paste. Tahineh is widely used in regions of the Middle East as a base ingredient for seasoned sauces and for hummus, a blended mixture of tahineh and chickpeas. Halawa is a thickened and sweetened tahineh-based dessert. Although the products are generally stored unrefrigerated in wholesale and retail outlets, the low water activity (a_w) of tahineh and halawa inhibits the growth of salmonellae but does not affect their survival in products stored for up to 4 months at ambient and refrigerator temperatures (D'Aoust, unpublished data). Contaminated tahineh and halawa imported into Canada originated from several manufacturers in Lebanon (Table 45–13). Subsequent monitoring of both sesame seed products in Canada and the US identified contaminated lots imported from several Middle Eastern countries. In considering the absence of published reports linking human salmonellosis with the consumption of these products, it should be recognized that for reasons discussed in Chapter 37, epidemiological investigations of outbreaks and sporadic cases do not always identify the causative food source.

45.5.2 Antibiotic Resistance

In addition to earlier considerations on the development of bacterial resistance and the widespread use of antimicrobial drugs in human medicine (Tables 45–3 and 45–4), the massive veterinary use and abuse of medically important antibiotics has

Table 45–12 Reported Incidence of *Salmonella* spp. in Raw Milk

		Milk Samples		*Number of Samples*		
Country	*Period*	*Source*	*Type*	*Tested*	*Percent Positive*	*Reference*
Canada	1985–1986	Milk lines	Filters	1,140	2.45	453
	1986–1987	Milk lines	Filters	1,873	0.53	452
	1995–1996	Bulk tanks	Fluid	1,720	0.17	623
England	1984–1987	Bulk tanks and local shops	Fluid	1,138	0.18	338
England and Wales	1992–1993	Bulk tanks	Fluid	1,673	0.36	483
France	ca 1996	Bulk tanks	Fluid	69	2.9	199
India	ca 1990	Local market	Fluid	69	0	597
Ireland	1989–1990	Bulk tanks	Fluid	589	0.16	546
United States	ca 1986	Delivery trucks	Swabs	523	5.4	455
		Delivery trucks	Fluid	155	2.6	
	1990	Bulk tanks	Fluid	292	8.9	565

contributed to the emergence and persistence of resistant salmonellae in the global food chain.[166,349,703] This deplorable situation stems from the lack of veterinary control on the use of antimicrobials in the treatment of diseased animals and, more importantly, in the widespread administration of subtherapeutic doses of antibiotics in feeds for growth promotion and for prophylaxis in meat animal husbandry. The international prevalence of antibiotic-resistant salmonellae in the poultry, porcine, and bovine meat industries is disquieting (Table 45–14). It is generally recognized that the widespread occurrence of streptomycin, tetracycline, and sulfa drug resistance in meat isolates results from the frequent agricultural use of these antibacterial agents at subtherapeutic levels.[176,349,703] The problem is amplified by the inability of slaughtering plants to significantly reduce the numbers of bacterial pathogens on meat carcasses and to effectively prevent cross-contamination within the plant environment.[703] The high levels of ampicillin resistance (≥80%) in meatborne *Salmonella* isolates from Brazil, France, and the US and the prominence of chloramphenicol and trimethoprim sulfamethoxazole-resistant salmonellae in raw meats from numerous countries are of great concern (Table 45–14). It has been reported that 20 (45%) of the 44 million pounds of antibiotics manufactured in the US in 1986 were used in the meat animal industry and that an estimated 90% of these antimicrobials were administered at subtherapeutic levels.[349] The use of prophylactic and growth-promoting antibiotic regimens and their role in the selection of resistant salmonellae in raw meat products is under review by the World Health Organization (Berlin 1997) and other national agencies.[330,493,619] The aquacultural industry is equally vulnerable to the emergence and persistence of antibiotic-resistant strains because it commonly uses antibiotic prophylaxis to safeguard the vigor of reared fish and shellfish.[176] The aqueous environment and the warm climates generally associated with aquacultural farms together with sedimentation of animal wastes and medicated feeds allow bacterial proliferation in earthen ponds and basins and the cross-contamination of reared species with resistant *Salmonella* strains.

The problem of antibiotic resistance is compounded by a natural propensity for the genetic transmission of resistance determinants via R-plasmids.[162,166] The conjugative intrageneric and intergeneric transfer of R-plasmids between compatible cells likely contributes to the high incidence of multiple antibiotic–resistant *Salmonella* strains in agricultural sectors (Table 45–14).[55,586] Antibiotic-resistance and virulence determinants can occur on a common plasmid and thus heighten public health concerns.[580,583,649] Several growth-promoting or prophylactic antibiotics such as apramycin, virgin-

Table 45–13 Reported Incidence of *Salmonella* in Domestic Fresh and Processed Vegetables

Food	*Number of Samples* Tested	*Number of Samples* Percent Positive	*Reference*
Bean Sprouts			
Malaysia (ca 1993)	10	20.0	44
Thailand (1990)	344	8.7	359
Cabbage			
Spain (1981–1983)	41	17.1	259
Carrots			
Spain (1981–1983)	42	7.1	258
United States (1979–1981)	95	1.1	570
Celery			
Spain (1981–1983)	26	7.7	259
United States (1979–1981)	48	2.1	570
Leafy Vegetables			
Greece (1978–1980)	157	1.9	500
Iraq (ca 1979)	43	7.0	7
Italy (1973–1975)	209	69.9	224
Malaysia (ca 1993)	24	4.0	44
Spain (1981–1983)	346	7.5	257
Spain (ca 1987)	65	7.7	281
The Netherlands (1976)[a]	69	5.8	635
United States (1979–1981)	41	2.4	570
Onion			
Spain (1981–1983)	45	4.4	258
Parsley			
Spain (1981–1983)	23	4.3	259
Radish			
United States (1979–1981)	95	1.1	570
Sesame			
Halawa			
Canada (1993)	35	17.5	D'Aoust (unpubl.)
Tahineh			
Canada (1993)	27	48.2	D'Aoust (unpubl.)
Egypt (ca 1980)	20	10.0	312
Saudi Arabia (1982–1983)	50	≤20.0	51
Tofu			
Malaysia (ca 1993)	8	12.5	44

[a] Domestic and imported product.

iamycin, tylosin, and avoparcin are widely used in animal husbandry and are regarded as veterinary drugs because of their limited therapeutic value in human medicine. However, the on-farm use of apramycin and avoparcin selects for meatborne bacteria resistant to the related and medically important gentamicin and vancomycin.[519,645,667] The bivalent resistance of *S.* Typhimurium to apramycin and

Table 45–14 Prevalence of Antibiotic Resistance in Nonhuman Isolates of *Salmonella*

				Antibiotic Resistance (%)[a]							
Country	*Period*	*Sample*	*Number of Strains Tested*	*A*	*C*	*TP[b]/SXT[c]*	*S*	*G*	*Cip*	*Multiple[d]*	*Reference*
Argentina	1993–1995	Chicken carcasses	93	21.5	8.6	9.7[c]	37.6	0.0	NL	52.7	640
Brazil	1990–1991	Raw meats	36	86.1	0.0	0.0[c]	94.4	75.0	NL	100.0	553
	ca 1993	Poultry feeds	29	46.5	17.9	NL[e]	100.0	17.9	NL	NL	74
Canada	1986–1989	Poultry	238	3.4	0.4	0.0[c]	48.7	5.0	NL	38.7	176
		Raw meats	92	10.9	5.4	0.0[c]	28.3	2.2	NL	21.7	
		Fish/shellfish	118	1.7	4.2	0.8	19.5	0.0	NL	7.6	
	1990–1991	Turkey/environment	2690	14.3	2.4	1.7[c]	NL	25.8	0	NL	527
		Layers/environment	457	2.9	0.2	0.4[c]	NL	0.4	0	6.3	525
		Broilers/environment	1159	2.0	2.0	0.9[c]	NL	7.7	0	21.2	
Former Czech Republic	1991–1992	Poultry	553	0.7	0.9	NL	5.4	NL	NL	6.1	129
France	1990–1992	Poultry	414	12.8	14.2	19.7[c]	10.4	0.5	NL	NL	579
	1994	Animal	100	100.0	91.0	10.0[c]	88.0	2.0	NL	100.0	115
Germany	1992	Chicken	245	2.4	4.7	6.2[c]	NL	NL	NL	NL	658
India	ca 1993	Meats	55	40.0	5.5	NL	29.1	9.1	NL	52.7	540
	ca 1994	Poultry/environment	65	6.2	4.6	0.0[c]	6.2	4.6	NL	100.0	537
	1993–1995	Fish	158	NL	7.6	NL	20.9	18.9	NL	NL	306
		Shellfish	82	NL	4.9	NL	17.1	11.0	NL	NL	
Japan	1988–1990	Animal environment	35	42.9	2.9	NL	88.6	NL	NL	57.1	465
Malta	1993	Bovine/fecal	131	0.8	0.8	3.1[b]	NL	0.0	0	NL	672
Morocco	ca 1995	Wastewater	90	41.1	16.7	66.7[c]	23.3	NL	NL	36.7	458
Nigeria	ca 1989	Bovine carcasses	23	21.7	13.0	NL	86.9	26.1	NL	NL	3
Thailand	1990	Bean sprouts	30	23.3	0.0	NL	3.3	0.0	NL	26.7	359
The Netherlands	1990–1991	Horses/fecal	53	39.6	32.1	26.4[c]	60.4	5.7	NL	52.8	668
United States	1988	Broiler carcasses	350	6.9	0.3	6.3[c]	44.3	22.2	NL	46.9	411
	ca 1990	Pork carcasses	121	80.1	11.6	80.2[b]	41.3	0.0	NL	93.3	223
	1990–1992	Poultry	467	83.0	4.0	2.0[c]	NL	5.0	1.0	87.0	475

[a] Ampicillin (A); chloramphenicol (C); streptomycin (S); gentamicin (G); ciprofloxacin (Cip)

[b] Trimethoprim

[c] Trimethoprim sulfamethoxazole

[d] Resistant to ≥ 2 antibiotics that may not be listed above

[e] Not listed

gentamicin is encoded on separate plasmids and stems from an acetyltransferase type IV–dependent inactivation of both aminoglycosides.[444,650]

Synthetic fluoroquinolones have been used increasingly in recent years for the treatment of systemic bacterial infections. Their efficacy derives from the inactivation of the active (A) site of the bacterial gyrase enzyme responsible for the stability and supercoiling of chromosomal DNA.[104] Bacterial resistance to fluoroquinolones may arise from a porin-dependent change in cell permeability or a mutation in the genomic *gyr A* locus encoding the gyrase subunit A protein[166,288,555] or as a result of gene-encoded alterations in the gyrase subunit B (binding) protein.[264,309] The therapeutic efficacy of the novel fluoroquinolones in human medicine is progressively being undermined by their increased prophylactic and therapeutic use in agriculture and aquaculture.[163,166] Fluoroquinolones originally were introduced to gap the therapeutic void created by the increase in bacterial resistance to other antibiotics (Tables 45–3 and 45–4). Since 1987, there has been a disquieting trend in government approval of the fluoroquinolones enrofloxacin in European countries[221,247,289,311] and sarafloxacin in the US[23] for the treatment of poultry and porcine infections. Complacency in promulgating proscriptive regulations on the use of fluoroquinolones in the food industry could bear devastating consequences on the safety of the global food supply and on the health of consumers. The incidence of bacterial cross-resistance to fluoroquinolones in veterinary isolates heightens public health concerns.[355,586] There are reports of 8.5% to 18.8% nalidixic acid resistance in *Salmonella* isolated from poultry and poultry feeds from Brazil,[74] France,[579] and The Netherlands,[355] of 3.2% to 12.9% enrofloxacin resistance in beef and chicken isolates of *S.* Typhimurium and *S.* Enteritidis in Germany,[658] and an increasing enrofloxacin resistance from 1.8% to 15.9% between 1988 and 1990 in bovine isolates in Germany.[311]

45.6 EPIDEMIOLOGY

Salmonella spp. are a leading cause of bacterial foodborne disease in most countries except Japan where *Vibrio* spp. dominate because of consumer preference for raw or lightly cooked fish and shellfish products (Table 45–15). The numerical data for a given pathogen are not directly comparable between countries because of national inequities in the reporting of diarrheal diseases and in the complete identification of infectious agents.[653,705] The data are nonetheless indicators of the true incidence of foodborne salmonellosis within individual countries. The ongoing pandemic of human *S.* Enteritidis infections has probably contributed significantly to the high numbers of foodborne *Salmonella* incidents in England and Wales, France, Italy, Spain, and Poland (Table 45–15). Dramatic increases in human foodborne salmonellosis (Figure 45–1) have been reported in recent years in France,[413,428,446,514] Austria,[705] Italy,[705] and England and Wales,[18,28,616] whereas the reported incidence continues to fluctuate in Scotland,[482] Spain,[207] Canada,[241,242] Israel,[705] and the US.[63]

Poultry, eggs, and egg products commonly were implicated in human *Salmonella* infections (Table 45–16). In spite of warnings from government and consumer protection agencies that the consumption of undercooked poultry and shell eggs and the addition of raw eggs to nonthermally processed foods are potentially hazardous, food preparators in food service establishments and private homes too often ignore this advice.[389,591,705] The growing popularity of raw meat dishes such as steak tartare, sushi, and the traditional light cooking of fish and shellfish indicate that consumer education on foodborne health risks remains a formidable challenge.[564,705] Although raw pork generally is well cooked to eliminate the threat of *Trichinella* parasites, neglect in the proper handling and cooking of this product can lead to human salmonellosis (Table 45–16). The use of raw or undercooked eggs in bakery products frequently is associated with *Salmonella* infections.[461,652] Raw milk remains an important vehicle of human *Salmonella* and other bacterial infections.[58,159,251,603] The health risks from the unrestricted retail sale of raw fluid milk in Scotland, Northern Ireland, England, and Wales were alleviated by proscriptive regulations in 1983–1985.[58] The retail sale of certified raw milk on the western coast of the US has also been associated with several episodes of human salmonellosis.[16,124,239] *Salmonella* outbreaks from pasteurized fluid milk were attributed to human error during milk processing or to postprocess contamination.[105,159,219,599] Properly controlled pasteurization eliminates vegetative bacterial pathogens from raw milk.[179,181,231] The debate concerning the enhanced flavor and nutritive value of raw milk cheese and the inherent bacterial health risk remains highly controversial. Major disease outbreaks from the consumption of raw milk cheese (Table 45–17) and the limited pathogen reductions during the mandatory 60-day refrigerated storage of cheese manufactured from unpasteurized milk clearly support the exclusive use of pasteurized milk in cheese produc-

Table 45–15 Epidemiology of Foodborne Bacterial Pathogens

		Annual Number of Reported Outbreaks								
Country	*Period*	*Salmonella* species	*S. aureus*	*C. perfringens*	*C. botulinum*	*Campylobacter* species	*Bacillus* species	*E. coli*	*Listeria* species	*Reference*
Canada	1991	25.0	18.0	0.0	1.0	5.0	8.0	7.0	0.0	654
England and Wales	1989–1991[a]	922.0[a]	8.3	50.4	0.3	5.0	24.7	1.0	0.0	705
Finland	1983–1990	7.8	5.9	9.3	NL[b]	1.9	3.6	0.3	NL	323
France	1993	826.0	37.0	32.0	NL	NL	3.0	NL	NL	413
Germany	1992	177.0	1.0	6.0	1.0	0.0	2.0	1.0	1.0	705
Hungary	1992	113.0	13.0	4.0	8.0	0.0	2.0	NL	NL	705
Italy	1991–1994	344.8	14.3	4.8	0.8	NL	1.0	0.5	0.3	592
Japan[c]	1991	159.0	95.0	21.0	3.0	24.0	9.0	30.0	NL	463
Poland	1994	453.0	8.0	NL	1.0	NL	NL	13.0	NL	534
Romania	1992	25.0	13.0	NL	2.0	NL	1.0	1.0	NL	705
Scotland	1980–1991[a]	162.7	1.5	6.2	NL	19.7	1.9	NL	NL	602
	1992	174.0	0.0	1.0	NL	11.0	0.0	0.0	NL	705
Spain (Catalonia)	1982–1990	30.3	2.0	0.3	0.2	NL	0.1	NL	NL	207
Spain (National)	1992	482.0	39.0	11.0	8.0	2.0	NL	7.0	NL	445
	1994	379.0	39.0	13.0	5.0	2.0	1.0	9.0	NL	510
Sweden	1990–1992	10.3	2.7	3.7	0.7	0.7	0.7	NL	NL	705
United States	1992	81.0	6.0	12.0	4.0	6.0	3.0	3.0	0.0	63

[a] Incomplete data for 1991.

[b] Not listed or possibly no reported outbreaks.

[c] A total 247 incidents of *Vibrio* spp. enteritis was reported.

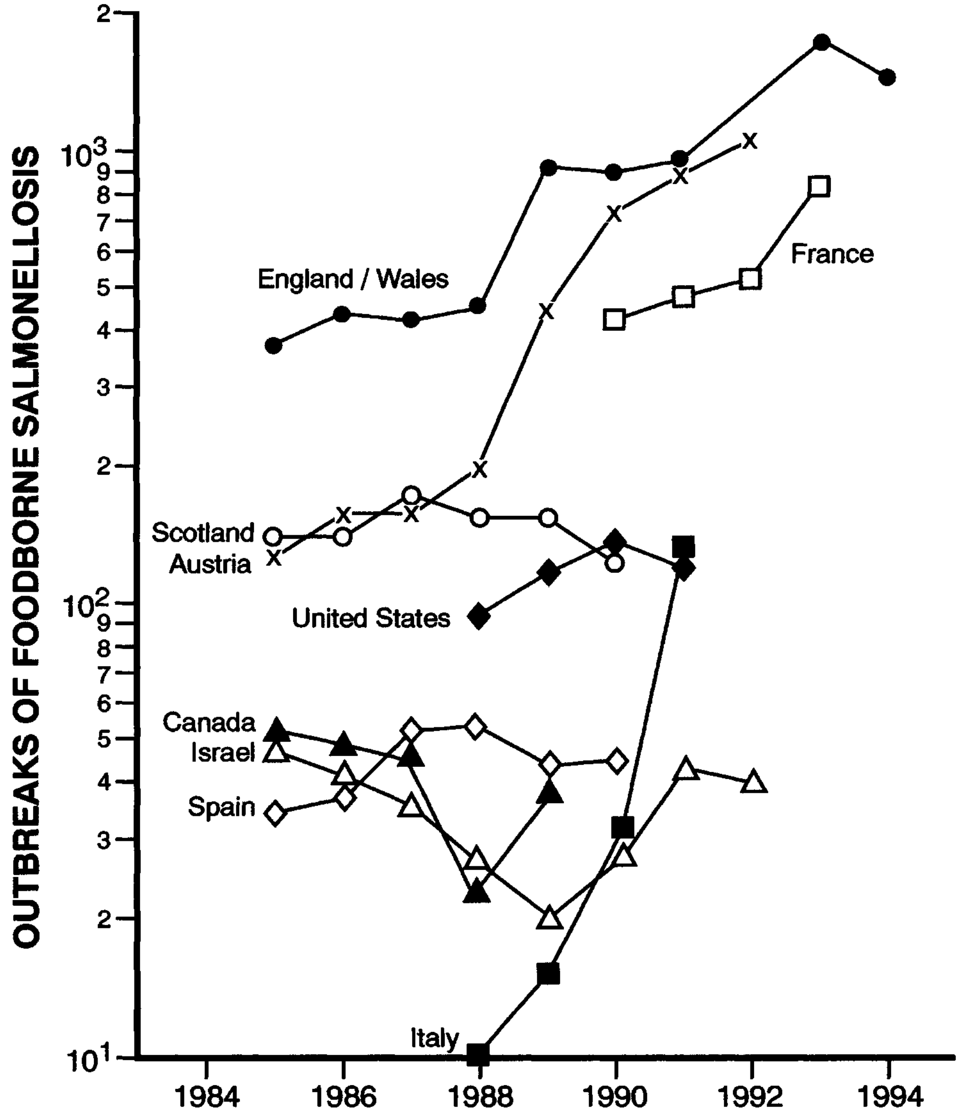

Figure 45–1. Trends in Reported Foodborne Salmonellosis

tion.[159,173,688,700] Fresh fruits and vegetables have gained notoriety in recent years as vehicles of human salmonellosis. The use of fresh or inadequately composted animal wastes as fertilizer, contaminated waters, and effluents for field irrigation, and nonpotable water for postharvest washing and production of ice for cold shipment of perishable products likely contributed to recent outbreaks attributed to fresh produce (Table 45–18). Alfalfa and mung bean seeds imported from Australia and The Netherlands were identified as the source of most sproutborne outbreaks.[437,536] Salmonellae may survive antibacterial treatments applied to seeds and can proliferate during seed germination.[82,356] The storage of freshly cut fruits at room temperature would favor the growth of salmonellae transferred from the rind of melons[164,280] or the surface of tomatoes[422,685,716] to edible tissues during slicing.

Dairy products figured prominently in the last decade as vehicles of major outbreaks of human salmonellosis (Table 45–19). Cheddar cheese made from pasteurized and thermized (unpasteurized) milk was adulterated following the manual override of a flow diversion valve, thereby channelling raw milk into vats of heat-treated cheese milk.[173] A single asymptomatic cow shedding the outbreak strain of *S.* Typhimurium PT10 from her right hind teat tentatively was identified as the source of contaminated

Table 45–16 Reported Number of *Salmonella* Outbreaks by Food Type

		Annual Number of Outbreaks[a]												
Country	*Period*	*Beef*	*Pork*	*Poultry*	*Unspecified Meats*	*Milk and Dairy Products*	*Egg and Egg Products*	*Vegetables and Salads*	*Fish and Shellfish*	*Bakery*	*Others*	*Unknown*	*Total*	*Reference*
Canada	1989	1.0	3.0	15.0	4.0	3.0	1.0	0.0	1.0	1.0	12.0	10.0	51.0	242
England and Wales	1989–1991	2.3	1.7	14.7	10.3	1.3	16.7	0.3	NL[b]	2.3	22.7	58.0	130.3	705
France	1993	NL	NL	NL	15.0	1.0	136.0	NL	4.0	NL	15.0	29.0	200.0	413
Germany	1991	NL	NL	6.0	8.0	4.0	28.0	2.0	2.0	NL	12.0	NL	72.0	713
Italy	1991–1994	NL	NL	4.3	13.8	2.8	182.0	NL	11.5	9.3	13.1	108.0	344.8	592
Japan	1991	NL	NL	NL	13.0	0.0	7.0	5.0	4.0	NL	51.0	58.0	138.0	463
Scotland	1989	0.0	1.0	20.0	4.0	0.0	10.0	NL	NL	NL	8.0	111.0	154.0	482
Spain (Catalonia)	1982–1990	NL	NL	0.0	1.1	0.0	10.2	1.2	0.6	1.3	1.4	14.6	30.4	207
Spain	1994	NL	NL	9.0	10.0	11.0	217.0	NL	5.0	24.0	25.0	77.0	378.0	510
United States	1992	0.0	0.0	0.0	0.0	1.0	5.0	6.0	0.0	3.0	20.0	45.0	80.0	63

[a] Mean annual number of outbreaks where applicable.

[b] Not listed.

Table 45–17 Bacterial Disease Outbreaks from Cheese Made with Unpasteurized Milk

Year	*Cheese*	*Cheese-Milk Processing*	*Country of Origin*	*Disease Agent*	Number of *Cases*	*Deaths*	*Reference*
1971	Camembert	Raw (putative)	France	*E. coli* O124 (EPEC)	387	0	443
1973	Goat cheese	Raw	Mexico	*B. melitensis*	3	0	216
1973	Brie	Raw (putative)	France and Switzerland	*C. botulinum* (type B)	75	0	593
1982	Accawi	Raw	Canada	S. Muenster	3	0	313
1983	Brie	Raw (putative)	France	*E. coli* O27 (ETEC)	169	0	431
1983	Queso Blanco	Raw	United States	*Strep. zooepidemicus*	16	2	228
1984	Cheddar	Thermized	Canada	S. Typhimurium	2,700	0	173
1985	Jalisco	Raw	United States	*L. monocytogenes* 4b	142	48	425
1985	Vacherin (soft)	Raw	Switzerland	S. Typhimurium	> 40	0	573
1989	Irish (soft)	Raw	England	S. Dublin	42	0	435
1990	Goat cheese	Raw	France	S. Paratyphi B	277	0	290
1992	Goat cheese	Raw	France	*E. coli* (VTEC not O157)	4	1	21
1993	Goat cheese	Raw	France	S. Paratyphi B	273	1	197
1994	Cheese	Raw	Scotland	*E. coli* O157	20	0	22
1995	Brie	Raw	France	*L. monocytogenes*	20	4	286
1995	Goat cheese	Raw	Malta	*B. melitensis*	135	1	24
1996	Cheddar	Thermized	England	S. Gold-coast	84	0	35

Table 45–18 Recent Outbreaks of Salmonellosis from Fresh Fruits and Vegetables

	Country			*Number of*		
Food	*Origin*	*Outbreak*	*Serovar*	*Cases*	*Deaths*	*Reference*
Alfalfa sprouts	Australia	Finland and Sweden (1994)	*S.* Bovismorbificans	492	0	521
	The Netherlands	United States and Finland (1995)	*S.* Stanley	242	0	437, 704
	The Netherlands	Canada and United States (1995–1996)	*S.* Newport	150	0	34a, 627
	Unknown	Denmark (1995)	*S.* Newport	>150	0	34
Cantaloupes	Mexico	United States (1989–1990)	*S.* Chester	>245	2	558
	United States	Canada and United States (1991)	*S.* Poona	>400	NS[a]	245
Mung bean sprouts	Australia	United Kingdom (1988)	*S.* Saint-paul	143	0	486
Mustard cress	The Netherlands	England and Wales (1989)	*S.* Gold-coast	14	0	362
Orange juice (unpasteurized)	United States	United States (1995)	*S.* Hartford *S.* Gaminara *S.* Rubislaw	63	0	27
Tomatoes	United States	United States (1990)	*S.* Javiana	>174	NS	701
Watermelon	United States	United States (1991)	*S.* Javiana	39	0	89

[a] Not specified

milk.[489] A massive outbreak of *S.* Typhimurium infection from pasteurized fluid milk resulted from a cross-connection between raw and pasteurized milk lines.[408] This episode remains the worst food poisoning incident in the US. The report of human *S.* Paratyphi B infections from unpasteurized goat milk cheese reiterates the inherent health hazard of raw milk.[198] Contamination of pasteurized ice cream mix during transport in trucks that previously had hauled raw liquid eggs led to a multistate outbreak of *S.* Enteritidis in the US.[314,315] A chilled beverage prepared with raw liquid eggs and served in a popular cafe that used unsanitary kitchen practices was incriminated in a large outbreak in the Republic of China.[709] Norwegian chocolate adulterated with *S.* Typhimurium led to disease outbreaks in Norway and Finland; the source of product contamination could not be ascertained.[377] A commercially distributed cooked egg dish ("nishikiito tamago") and spiced Italian-style prosciottino ham purchased from delicatessens and restaurants caused major outbreaks of salmonellosis in Japan and the US, respectively.[19,463] Fresh cantaloupes produced in the southern US were linked epidemiologically to nationwide outbreaks of *S.* Poona in Canada and the US.[245] A contaminated mixture of flaked tuna and raw egg mayonnaise prepared in a central kitchen and served in school cafeterias led to an outbreak of *S.* Enteritidis that affected 751 individuals, many requiring hospitalization.[556] Contaminated paprika sprinkled onto nationally distributed potato chips was implicated in a major episode of human salmonellosis in Germany.[412] In addition to the three principal serovars isolated from patients, paprika imported from South America, and paprika potato chips (Table 45–19), a total of 94 salmonellae including newly identified serovars and unusual strains capable of growth in KCN were recovered during the outbreak period. A disease outbreak of *S.* Bovismorbificans in Finland and Sweden was attributed to alfalfa seeds imported from Australia and germinated in different sprouting facilities in both Scandinavian countries.[521] The increasing movement of food in international trade, the national disparities in quality control programs and safety standards, and overextension of inspection and analytical capabilities in importing countries likely contributed to international outbreaks of foodborne salmonellosis (Table 45–20).

45.7 FACTORS AFFECTING GROWTH AND SURVIVAL IN FOODS

45.7.1 Growth

Mesophilic *Salmonella* have been reported to grow in the temperature range of 2° C to 47° C and up to 54° C for a thermally stressed mutant of *S.*

Table 45–19 Major Foodborne Outbreaks of Human Salmonellosis

Year	Country	Vehicle	Serovar	Number of Cases[a]	Number of Deaths	Reference
1984	Canada	Cheddar cheese	S. Typhimurium PT 10	2,700	0	173
1985	United States	Pasteurized milk	S. Typhimurium	16,284	7	408
1987	Republic of China	Beverage (egg)	S. Typhimurium	1,113	NS[b]	709
1987	Norway and Finland	Chocolate	S. Typhimurium	361	0	377
1988	Japan	Cooked eggs	*Salmonella* spp.	10,476	NS	463
1991	United States	Prosciottino ham	*Salmonella* spp.	> 350	NS	19
1991	United States and Canada	Cantaloupes	S. Poona	> 400	NS	245
1993	France	Mayonnaise	S. Enteritidis	751	0	556
1993	Germany	Paprika chips	S. Saint-paul S. Javiana S. Rubislaw	1,000	0	412
1993	France	Goat cheese (raw)	S. Paratyphi B	273	1	198
1994	United States	Ice cream	S. Enteritidis	740	0	314
1994	Finland and Sweden	Alfalfa sprouts	S. Bovismorbificans	492	0	521

[a] Confirmed cases.
[b] Not specified.

Typhimurium.[160,162,166,211,212] The ability of salmonellae to proliferate in chill-stored foods emphasizes the importance of adequate refrigeration and rapid turnover of perishable foods. The latter increasingly are being packaged under vacuum or modified atmospheres consisting of 60% to 80% CO_2 with varying proportions of N_2 and/or O_2 to prolong shelf-life through the inhibition of aerobic spoilage microorganisms.[128,162] In fact, modified atmosphere packaging (MAP) removes these natural organoleptic indicators of temperature abuse of the product. Populations of *Salmonella* can remain unchanged at high concentrations of CO_2 but rapidly multiply in foods packaged under 20% to 50% CO_2.[77,348] Preconditioning at low temperatures can increase the growth rate of *Salmonella* at low temperatures and the minimum temperature for growth may be lower in foods than in laboratory media.[6,160,162] The minimum temperature for growth prevails at neutral pH and increases sharply as pH deviates from neutrality.[448] Salt concentrations of ≥ 2% (w/v) reportedly delay the growth of salmonellae.[454] The proliferation of salmonellae at $a_w \geq 0.93$ varies between strains and depends on the composition of the food.[160] Refrigerator temperatures amplify NaCl-dependent bacteriostasis whereas increasing temperatures increase bacterial salt tolerance.[9,643] For example, the maximum NaCl concentration for the growth of S. Typhimurium in brain heart infusion broth (pH 6.8) at 10° C and 15° C was 2% and 6%, respectively.[269] Salt-dependent reductions in growth rate may result from altered enzyme configurations and reaction kinetics or from the expenditure of cellular energy to maintain ionic homeostasis. *Salmonella* spp. grow at moderate temperatures (25° C to 43° C) in the pH range of 3.6 to 9.5 with an optimum pH of 6.5 to 7.5.[49,127,329,572] The minimum pH for growth was determined in a defined broth medium acidified with HCl.[572] The antibacterial action of acetic and propionic acids exceeds that of the naturally occurring citric acid or that of lactic acid produced during the fermentation of meat and dairy products.[127,160,454,572] Slow acidification during fermentative processes likely encourages pH adaptation and growth of salmonellae under normally inhibitory acidic conditions.[337] Acid tolerance at pH ≤ 5.0 increases with increasing temperature.[9,238,643] A growth study of *Salmonella* on NaCl-pH gradient plates varying from 2.7% to 10% NaCl and pH 3.76 to 7.9 confirmed the primacy of pH as growth determinant.[643] Interestingly, supplementation of complex and defined broth media with 2% (w/v) NaCl or KCl and adjustment to pH 5.2 with acetic acid showed low salt-dependent growth stimulation of S. Enteritidis at 25° C and 37° C.[538] The protection afforded by low salinity may be linked to the Na^+/K^+-proton antiport system and maintenance of cellular homeostasis.[166] Predictive (stochastic) modelling allows food manu-

TABLE 45–20 *Salmonella* Outbreaks from Imported Foods

	Country				*Number of*		
Year	*Exporting*	*Importing*	*Vehicle*	*Serovar*	*Cases*	*Deaths*	*Reference*
1973	Canada	United States	Chocolate	S. Eastbourne	122	0	146, 177
1982	Italy	United Kingdom	Chocolate	S. Napoli	245	0	274
1982	Brazil	Norway	Black pepper	S. Oranienburg	126	1	297
1984	France	United Kingdom	Pâté	S. Gold-coast	506	0	94
1984	United Kingdom	International	Aspic glaze	S. Enteritidis PT 4	766	2	110
1985–1986	Belgium	Canada	Chocolate	S. Nima	29	0	324
1987	Norway	Finland	Chocolate	S. Typhimurium	12	0	377
1988	Australia	England	Mung bean sprouts	S. Saint-paul	143	0	486
1989	The Netherlands	England	Mustard cress	S. Gold-coast	14	0	362
1989–1990	Mexico	United States	Cantaloupe	S. Chester	> 245	2	558
1994	Australia	Finland and Sweden	Alfalfa sprouts	S. Bovismorbificans	492	0	521
1995	The Netherlands	United States and Finland	Alfalfa sprouts	S. Stanley	> 230	0	627, 704
1995–1996	The Netherlands	Canada and United States	Alfalfa sprouts	S. Newport	150	0	34a, 627
1996	France	United Kingdom	Infant milk formula	S. Anatum	> 12	0	36

facturers to estimate the shelf-life and safety of products. The models are mathematical derivations that delineate the probable growth or survival response of foodborne bacteria under different combinations of pH, temperature, salt concentration, a_w, or other environmental factor of interest (Chapter 16[270,318,567]). The predictive power of this approach stems from the development of a large database on bacterial responses in broth media or food matrices under defined and controlled test parameters. These responses are then fitted to mathematical equations which, by interpolation, putatively predict the behavior of salmonellae or other bacterial species under particular environmental conditions. Predictive models are not without limitations.[270,567] Their application to conditions outside the boundaries of the experimental database could lead to erroneous conclusions. The physiological heterogeneity within the *Salmonella* genus undermines the reliability of models based on data from a few serovars. The types and numbers of endogenous microflora in foods could also affect the growth and survival of salmonellae but this variable has yet to be adequately factored into predictive models. The different behavior of microorganisms in liquid and on solid media also dictates caution in the use of predictive models derived from bacterial responses in aqueous environments.[101,102] The field of predictive modelling is rapidly expanding and striving to address the fate of bacterial pathogens in complex food systems,[213,689] under fluctuating environmental conditions,[565] and in different physiological states.[318] Predictive models are commercially available in the UK and the US in the form of the Food Micromodel and the Pathogen Modeling Programs, respectively.

45.7.2 Survival

The potential survival of *Salmonella* under extreme environmental conditions is a major public health concern. The fate of salmonellae during freezer storage is strain- and food-dependent, where the number of viable cells gradually decreases during storage at –20° C or lower with more rapid death at near-freezing temperatures.[57,160,166] *Salmonella* have survived in ice cream and raw snails stored for many years at –20° C or lower.[160] Survival also hinges on the physiological state of the microorganism prior to freezing.[80] Short (30 min) adaptation of *S.* Enteritidis to low temperatures (5° C to 10° C) prior to rapid freezing at –78° C greatly enhanced survival. Cold temperature adaptation induced the synthesis of a genomic *csp* A-encoded cold shock protein whose specific role in freezer protection remains undetermined.[358] Although the low a_w in dry foods inhibits the growth of salmonellae, it also triggers bacterial stress responses that promote greater survival and potentiate outbreaks of human salmonellosis, (Tables 45–19 and 45–20).[160] Survival in dry products is dependent on a_w, temperature, and food type. An increase in the moisture content of dry products including milk powder, cocoa powder, and animal feeds stored at room temperature or storage of dry foods at an elevated temperature (>25° C) enhances bacterial lethality.[56,373]

Salmonella can survive in low pH environments such as naturally acidic, acidified, and fermented foods.[160,415,502,659] This is of particular concern because, in some circumstances, the infectious dose for humans can be low.[164] Several strains of salmonellae were inactivated within hours in sweet pickle relish (pH 2.8) and mustard (pH 3.1) but survived 24 hours in ketchup adjusted to pH 3.6.[659] Similarly, acid-conditioned (pH 5.0) *Salmonella* inocula survived for 27 and 73 days in orange juice adjusted with citric acid to pH 3.5 and 4.0, respectively.[502] A mixture of *Salmonella* in a commercial reduced-calorie mayonnaise was rapidly inactivated as the pH was adjusted from 4.3 to 3.9 with acetic acid.[277] Acidification of mayonnaise with acetic acid was markedly more effective than citric acid at pH values of 4.8 to 5.2 in reducing levels of *S.* Muenster during storage at 25° C.[137] Studies on the decontamination of meat carcasses have also confirmed the greater lethality of acetic acid.[202] In contrast to cold temperatures, which promote bacterial survival in mayonnaise, holding this food at room or higher temperatures more effectively inactivates *Salmonella* spp.[426,481,507,659] The identity of vegetable oil used in the preparation of mayonnaise also influences survival of *Salmonella*.[539]

A constitutive pH homeostasis system maintains the cytoplasm of *Salmonella* cells at pH ≥ 6.0 under mild to moderate environmental acid conditions (pH > 4.0). Control mechanisms include the low permeability of the cytoplasmic membrane to protons, a proton translocating ATPase that pumps cytoplasmic H^+ into the cell exterior, the H^+ and Na^+/K^+ antiport system that exchanges cytoplasmic H^+ with monovalent cations from the external medium, and the electron transport chain that converts protons into water. Acid-induced bacterial decarboxylases also contribute to pH homeostasis by generating amines that neutralize H^+ in the cytoplasm.[244,317,491] These mechanisms fail under extreme acidic conditions, resulting in bacterial cell death.

The physiological state of salmonellae as affected by growth conditions has an impact on acid resistance. Growth of *S.* Enteritidis at 37° C to 44° C induced greater acid resistance (pH 2.6) than growth at 20° C. A sudden temperature shift from 20° C to 37° C–46° C triggered a similar bacterial response.[342] Acid resistance can also be enhanced if salmonellae first are adapted to moderately acid conditions (pH > 4.3) prior to their exposure to otherwise bactericidal conditions (pH 3.0 to 3.3). The phenomenon termed acid tolerance response (ATR) hinges on the synthesis of acid shock proteins.[243] Different ATR systems are activated in the logarithmic and stationary phases of growth. Pre–acid-shock adaptation (pH 5.8) and acid shock adaptation (pH 4.3) both have been used to induce ATR in log phase cells. In the pre–acid-shock adaptation, salmonellae are maintained in minimal glucose broth medium at pH 5.8 for one-cell doubling prior to acid challenge at pH 3.0 to 3.3. The acquired resistance results from an induced elevation in cytoplasmic pH that permits *Salmonella* spp. to synthesize protective acid shock proteins under normally inhibitory acid (pH 3.0 to 3.3) conditions. The alternate sigma factor (σ^s) encoded by the *rpo S* locus is an essential element in this protective response. Acid shock adaptation in cells held for ≤ 60 minutes at pH 4.3 can lead to either transient or sustained responses in log phase salmonellae. Although the induction of the transient ATR is independent of the alternative sigma factor (σ^s), the response is modulated by the gene product of the *fur* iron regulator locus, which controls the expression of several acid shock proteins.[243] In contrast, the sustained ATR is σ^s-dependent and more rigorously protects log phase cells against the acid environments. The acid shock (pH 4.3) also induces a rapid four- to five-fold increase in cellular levels of σ^s that specifically directs the synthesis of 8 of the 51 acid-shock proteins associated with the ATR in log phase salmonellae. High levels of σ^s alone do not confer resistance unless cells also are subjected to an acid shock. Although the underlying protective mechanisms have yet to be fully elucidated, the ability of salmonellae to efficiently repair acid-damaged DNA plays a determinant role in bacterial survival. Two ATRs associated with salmonellae in the stationary phase of growth engender the synthesis of up to 15 acid shock proteins.[243] A general stress response is induced when salmonellae enter the stationary phase of growth. This σ^s-dependent system is not acid inducible. In contrast, a second stationary-phase ATR is acid inducible but σ^s-independent. The protective capacity of these responses greatly exceeds that afforded in log phase ATRs. Current evidence suggests that the acidity of the extracellular and cytoplasmic environments induces the synthesis of different sets of protective acid shock proteins. Induced acid tolerance also engenders resistance to thermal, osmotic, and oxidative stress conditions.[243,416] Conversely, heat stress affords cross-protection against normally lethal acidic microenvironments.[342]

Salmonella poorly withstand exposure to pH >10.0, which disrupts the cytoplasmic membrane.[118,329,388,456] Synergism between high alkaline pH and elevated temperatures increases the death rate.[497,639]

The acquired or induced thermoresistance of *Salmonella* underlines the importance of commercial heat processes in providing safe and wholesome foods. The greater heat tolerance of salmonellae in the stationary than logarithmic phase of growth and in cells preconditioned at sublethal temperatures (≤50° C) or grown in nutritionally rich media derives, in part, from the synthesis of heat shock proteins and/or alteration of cytoplasmic membrane fluidity.[108,118,166,342,636] A few highly heat-resistant strains such as *S.* Senftenberg 775W[391,401,480] and *S.* Irumu[81] have been reported. Heat resistance frequently is expressed in terms of decimal reduction time (*D*), which represents the amount of time required at a stated temperature to effect a 1.0 $\log_{10}$ reduction in the number of viable microorganisms. The heat resistance of *Salmonella* varies inversely with the a_w of the heating menstruum,[160,628] and is also modulated by the nature of low molecular weight solutes therein.[140,160,391,496] For example, sucrose adjusted to a_w = 0.90 confers greater heat resistance to *S.* Typhimurium at 57.2° C than glycerol at the same a_w value.[279] Although the mechanism for a_w- and solute-dependent increase in heat resistance remains uncertain, induced plasmolysis could trigger structural alteration of enzymes to heat-resistant configurations.[160] Heat resistance tends to be highest at pH values approaching neutrality.[497] Thermal inactivation models were recently developed for *S.* Enteritidis using temperature (54.5° C to 64.5° C), pH (4.2 to 9.6), and NaCl (0.5% to 8.5%) as controlling factors.[86]

Standard procedures for the pasteurization of egg yolk products in the US include heating of liquid egg yolk for 3.5 minutes at 61.1° C, egg yolk with 10% NaCl, 10% sucrose, or 10% NaCl plus 5% sucrose for 3.5 minutes at 63.3° C, and egg yolk with 20% NaCl for 3.5 minutes at 64.4° C.[496] The *D* (61.1° C) values for mixtures of *S.* Enteritidis and *S.* Typhimurium inoculated into plain yolk ranged from 0.16 to 0.57 minutes, which correspond to 6.14 to 21.9 $\log_{10}$ reductions in a standard 3.5-minute pasteurization process.[496,590] The heat resistance of test strains increased in the presence of a_w-reducing solutes. For example, the *D* (63.3° C) value of a mixture of *Salmonella* strains increased from 0.20 minutes in liquid egg yolk to 0.72 and 11.50 minutes in egg yolk supplemented with 10% sucrose and 10% NaCl, respectively.[496] The modest 0.3 $\log_{10}$ reduction obtained with the standard 3.5 minutes processing of salted products at 63.3° C indicates that this thermal process would not confer product safety for raw egg yolks containing 10% NaCl and high incident levels of salmonellae. Temperature upshifts also can increase bacterial heat resistance. The *D* (56° C) value of *S.* Enteritidis phage-type 4 grown at 20° C was 0.91 minutes, and 14.4 minutes for bacteria grown at 44° C.[342] The use of wash-water tanks held at 32° C to 46° C to sanitize eggshell surfaces and the subsequent slow cooling of washed shell eggs may induce heat-shock protein synthesis and enhance bacterial heat resistance.[368,430]

Commercial thermal processing of raw eggs plays a key role in the bacteriological safety of derived products particularly in relation to *S.* Enteritidis (see Section 45.5.1). Although an early report suggested that *S.* Enteritidis phage-type 4 was highly thermotolerant,[341] subsequent evidence suggests that this and other phage-types of *S.* Enteritidis are not unduly heat-resistant.[496,590,596] The pasteurization of liquid egg white presents a major challenge because its desirable functional properties are destroyed rapidly at elevated temperatures.[497] The transovarian transmission of *S.* Enteritidis into shell eggs and the frequent localization of this pathogen in egg albumen emphasize the importance of effective liquid egg pasteurization.[341,344] Parameters for the pasteurization of liquid egg white were developed when the pH of the raw products ranged from 8.9 to 9.1. The currently more efficient movement of shell eggs from commercial layer farms to egg-breaking stations now provides egg albumen with a pH close to 8.2.[497] The resulting reduction in the pH of whole egg melange[15] or liquid egg white[260,497,590] dramatically increases the heat resistance of *Salmonella* spp. These concerns led to a reassessment of the lethality of conventional heating regimens for liquid albumen which include 3.5 minutes at 51.7° C in the presence of H_2O_2, 6.2 minutes at 55.6° C, and 3.5 minutes at 56.7° C.[662] Using a submerged vial technique and mixtures of *S.* Enteritidis, *S.* Typhimurium, and *S.* Senftenberg, none of the foregoing heat processes achieved the minimum 4.0-log reduction in viable salmonellae.[497] Similarly, pooled strains of *S.* Enteritidis and *S.* Typhimurium inoculated into albumen (pH 8.2) and heated in sealed glass vials yielded a *D* (56.7° C) value of 2.96 minutes which corresponds to a 1.18 $\log_{10}$ reduction in a standard 3.5 minutes pasteurization process.[590] Lower heat resistance was observed in albumen (pH 9.1) with *D* (56.7° C) value of 1.58 minutes.

Pasteurization of raw milk at 71.7° C for 0.25 minutes or under equivalent time-temperature regimens targets a 5.0 $\log_{10}$ or greater reduction in the number of viable salmonellae.[663] *D* values of 0.11 minutes (62.8° C) and 0.004 minutes (71.7° C) were obtained for the *S.* Typhimurium strain associated with the largest milkborne outbreak (1985) in the US. It was suggested that the outbreak resulted from postprocess contamination of milk rather than deficiencies in the proper operation of the pasteurizer.[97] Thermization is widely used in the cheese industry and involves the holding of raw milk for short (nonpasteurizing) periods of time at temperatures ranging from 60° C to 69° C. In a pilot plant study on the fate of *Salmonella* spp. in thermized cheesemilk,[179] the *D* values for 10-strain mixtures of salmonellae inoculated into raw milk and heated for 0.27 to 0.29 minutes at 60° C in a high temperature, short time (HTST) pasteurizer ranged from 0.058 to 0.098 minutes, which correspond to a 3.0 to 5.0 $\log_{10}$ reduction in viable salmonellae. Thermal processing of these strains at 63° C or greater generally produced more than 6.0 $\log_{10}$ reductions. In contrast, the heat-resistant *S.* Senftenberg 775W required treatment at 67.5° C to effect a 6.0 $\log_{10}$ reduction.[179] The foregoing considerations suggest that thermization of heavily contaminated raw milk could lead to hazardous dairy products.[159] The heat-resistance of *Salmonella* spp. also increases with increasing concentrations of milk solids but decreases when the test strain is heated under reduced atmospheric pressure.[195]

45.8 PRINCIPLES OF DETECTION AND ISOLATION

45.8.1 Cultural Methods

The use of statistically significant sampling plans and sensitive analytical procedures are prerequisites for the successful identification of *Salmonella*-contaminated foods and agricultural products. The sampling plans of the International Commission on Microbiological Specifications for Foods (ICMSF) are based on the perceived degree of hazard associated with the distribution and consumer handling of a food, and on the desired stringency in assessing product safety.[351] Sampling plans define the number of replicate samples (n) to be taken at random from a production lot and the acceptance criterion (c), which corresponds to the maximum number of positive replicate samples that would be tolerated and not engender lot rejection. A zero tolerance ($c = 0$) for *Salmonella* has been proposed for all food categories.[351] *Salmonella* testing is a qualitative decision process that corresponds to a two-class attribute sampling plan whose stringency can be increased by adjusting the value for n and/or c. The use of analytical units greater than the 25g portions recommended in conventional methods also would increase stringency through enhanced method sensitivity.[14,168,240,350] Dry or wet compositing can markedly reduce the workload in the analysis of replicate samples from one or more lots of product.[160] In dry compositing, up to 15 × 25g analytical units can be combined into a single test. Thus, a lot of product examined with a stringency of $n = 60$ would be represented by a total of four test composites. A positive composite would signify that one or more of the pooled units was contaminated. Identification of the adulterated unit(s) within the positive composite would rest on the repeated analysis of individual units. In wet compositing, no more than 10 × 1 ml portions of preenrichment cultures should be combined into a single test for enrichment in a selective broth medium. The consecutive use of dry and wet compositing is not recommended because it would likely undermine method sensitivity.[157,160]

Conventional cultural methods for the detection of foodborne *Salmonella* consist of five distinct analytical steps.[14,168,240,350] These include nonselective enrichment (preenrichment) and selective enrichment in broth media, isolation of suspect *Salmonella* colonies on differential agar media, biochemical screening, and serological confirmation of isolates. Preenrichment allows the resuscitation of low numbers of stressed or injured salmonellae.[160,165] All test materials should be preenriched, including samples suspected or known to contain high levels of background microflora. The equivalence of buffered peptone water (BPW),[168,350] lactose,[14,240] and nutrient[168] preenrichment broths to more complex sterility testing media suggest that the nutrient requirements for successful preenrichment are minimal.[157,167] The choice of lactose broth for the preenrichment of commonly lactose-negative *Salmonella* strains remains paradoxical. Preenrichment in lactose broth in which the final pH of the preenrichment culture was 4.4 gave a lower recovery of salmonellae from cheese than preenrichment in a similar non-lactose containing medium in which the final pH was 5.7.[326] These findings contrast with the equivalence of nonbuffered lactose broth and BPW in the detection of salmonellae in raw egg products, frozen vegetables, and animal feed where the final pH of the lactose and BPW preenrichment cultures ranged from 3.8 to 5.9, and 4.8 to 6.9, respectively.[160] Although the progressive acidification of lactose preenrichment cultures to pH ≥ 3.8 is bacteriostatic and potentially lytic, the concomitant induction of the acid tolerance response (Section 45.7.2) would permit salmonellae to grow to detectable levels.

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Levels of 10^0 to 10^7 salmonellae ml^{-1} in overnight lactose and nutrient broth cultures reflect strain-specific responses and synergistic or antagonistic effects of food components and competitive microorganisms.[160,167] Surfactants including Tergitol-7 and Triton x-100 are used for the preenrichment of fatty foods.[14,175] The surfactants putatively liberate salmonellae from lipid micelles, allowing their proliferation in the aqueous phase; however, it has been suggested that the use of surfactants could lead to erroneous results arising from surfactant toxicity.[175] Preenrichment at elevated temperatures (41° C to 43° C) is not advisable because it introduces an additional stress under conditions designed to resuscitate debilitated *Salmonella* cells.[160,695] Attempts at cultural method brevity through short (6 to 8 hours) preenrichment have proven unsuccessful.[160,300] Short preenrichment does not favor bacterial proliferation and likely results in the transfer of low numbers of poorly resuscitated salmonellae into selective (toxic) enrichment media.[84,157,160,300] Prolonged (>24 hour) preenrichment may adversely affect the detection of foodborne salmonellae.[160,339] The volume of overnight preenrichment culture inoculated into selective enrichment broth did not alter method sensitivity where transfer volumes of 10.0, 1.0, and 0.1 ml were equally effective in detecting naturally contaminated foods and animal feeds.[157,160] However, with short (6 hour) periods of preenrichment, detection was transfer volume–dependent.[167] The use of 10 ml transfer volumes[350] does not improve method performance and, compared with other standard methods,[14,168,240] increases 10-fold the required volume of costly enrichment media. Direct enrichment has been applied to foods and agricultural products known or suspected to contain high levels of background microflora. The suspension of such materials into selective enrichment media (no preenrichment) was meant to repress the growth of nonsalmonellae and favor the outgrowth of salmonellae through reduced competition for nutrients. However, the low sensitivity of this approach seemingly stems from the direct contact of debilitated salmonellae with selective (toxic) conditions resulting in the death of the few *Salmonella* cells that frequently contaminate test materials.[157,160,624]

The enrichment of samples in selective broth media favors the growth of salmonellae through the inhibition of competitive microorganisms. Enrichment media including tetrathionate brilliant green (TBG), Rappaport-Vassiliadis (RV), and selenite cystine (SC) figure prominently in standard methods of analysis.[14,168,240,350] The use of the Hajna, Rolfe, and the two Mueller-Kauffman (North American and European) formulations of TBG, each with distinct levels of sensitivity, specificity, and buffering capacities warrants caution in the interpretation and interstudy comparison of experimental results. The selectivity of TBG stems from the tetrathionate ($S_4O_6^=$) anion formed from the reaction of thiosulfate ($S_2O_3^=$) with iodine. The molarity of $S_4O_6^=$ in the different TBG formulations ranges from 0.016 to 0.043.[157] Membrane-bound tetrathionate reductase in *Salmonella*, *Citrobacter*, *Serratia*, and *Proteus* affords protection against the $S_4O_6^=$-dependent inhibition of protein synthesis and the inactivation of active sites of enzymes.[160] The regular quality control of this and other selective and potentially toxic media is essential. The RV and TBG enrichment media are closely related in their use of RV and TBG triphenylmethane dyes as selective agents and their incubation at elevated (41° C to 43° C) temperatures. High concentrations of $MgCl_2$ further increase RV selectivity; however, the dependence of RV on a 1:100 transfer volume for the optimal recovery of foodborne salmonellae underlines the limited selectivity of this medium.[160,509,656,669,670] SC is a medium of low selectivity that is nevertheless important in the recovery of fastidious salmonellae that do not grow or grow poorly in TBG and RV media.[158,160] The selectivity of SC hinges on the more rapid uptake of selenite ($SeO_3^=$) by competitive microflora and the incorporation of inactivating seleno-polythionate analogs of sulfur-containing amino acids into enzyme proteins.[614] Growing human health concerns on the safe handling and disposal of SC are leading to its replacement with other enrichment media.[14] The incubation of TBG and RV at selective (41° C to 43° C) rather than permissive (35° C to 37° C) temperatures markedly increases method sensitivity by facilitating isolation of *Salmonella* on plating media.[157,160,372,605] SC is generally incubated at 35° C to 37° C because selenite-based media can be inhibitory to *Salmonella* at higher temperatures.[43,160] Comparative studies have ranked 16 to 24 hours enrichment conditions in the following decreasing order of effectiveness: $TBG_{43} \geq RV_{43} > TBG_{35} > SC_{35}$.[10,54,87,160,305,371,455,605] A minimum of two enrichment media and two incubation temperatures always should be used to optimize the detection of contaminated samples. For example, a combination of TBG_{43}/SC_{35} would be preferable to TBG_{43}/TBG_{35} or TBG_{43}/RV_{43} because it provides greater physiological latitude for the enrichment of *Salmonella* strains that poorly tolerate elevated temperatures or do not grow in media containing malachite green or brilliant green (BGA) dyes. The value of SC_{35} was evidenced in a major collaborative study where the medium uniquely identified 5.5% of 1,616 naturally contaminated foods and agricultural products.[175] The use of abbreviated (6 to 8 hours) enrichment has met with limited success because the approach undermines the full selective potential of enrichment media.[10,160,172] Prolonged incubation (≥ 48 hours) of enrichment media does not necessarily increase method sensitivity.[54,160]

Enrichment cultures are plated on differential and selective agar media that respond principally to the bacterial release of hydrogen sulfide or to acid production from discriminating substrates.[14,160,168,240,350] These agar media include BGA, brilliant green sulfa (BGS), xylose-lysine-desoxycholate (XLD), and Hektoen enteric (Hek) agars that respond to lactose and/or sucrose utilization.[160] The low selectivity of these media can be a problem because competitive strains can mimic *Salmonella* and result in costly and futile tests on suspect isolates. The occurrence of lactose- and/or sucrose-positive salmonellae limits the value of saccharide-dependent plating media.[160] Bismuth sulfite (BiS) agar is used widely because of its highly sensitive H_2S-detecting system and saccharide-independent differentiation of isolates.[156,160] Novel plating media include xylose-lysine-Tergitol-4 (XLT-4),[459,605] which is XLD with added Tergitol-4 for increased selectivity, Rambach agar,[268,365,369,396,512,543,605] and the ready-to-use SM-ID.[184,365,512] In addition to β-galactosidase activity, the Rambach and SM-ID media show acid production from propylene glycol and glucuronate, respectively. Suspect *Salmonella* colonies generally develop within 16 to 24 hours at 35° C to 37° C except on BiS, which may require 48 hours of incubation.[156,160,165] Reports on the sensitivity and specificity of plating media support the following ranking in decreasing order of effectiveness : BiS > BGS > BGA ≥ Rambach = SM-ID = XLT-4 > XLD > Hek.[160,184,605,680] The physiological diversity within the *Salmonella* genus necessitates the use of two or more plating media for the effective detection of the target microorganism in enrichment cultures.[158,160,334,680] The use of lactose and/or sucrose, the production of H_2S, and presence of lysine decarboxylase and urease are key determinants in the biochemical screening of presumptive *Salmonella* isolates.[14,168,350] Commercially available biochemical kits have proven invaluable in the identification of atypical salmonellae and taxonomically related microorganisms.[14,145,160] Nonetheless, confirmation of a presumptive *Salmonella* isolate rests on somatic (O) and flagellar (H) agglutination reactions using polyvalent and single grouping antisera. In contrast to somatic antisera, which may produce false-positive reactions with closely related nonsalmonellae, the highly specific flagellar (H) antisera provide for the conclusive identification of *Salmonella* strains.

Several cultural approaches promote the more rapid screening of *Salmonella*-negative samples. The Salmosyst® system combines nonselective and selective enrichment into a single analytical step through the progressive release of selective agents in the preenrichment broth culture. Following overnight incubation at 35° C, the enriched culture is inoculated onto appropriate plating media. Presumptive positive or negative results are thus obtained 24 hours earlier than with conventional cultural methods. Reported levels of agreement between the Salmosyst® and standard cultural procedures vary widely.[511,513,683]

The RV enrichment broth was reformulated to the modified semisolid Rappaport-Vassiliadis (MSRV) plating medium by increasing nutrient levels and selectivity through the addition of novobiocin.[191] Synergism between novobiocin, malachite green, acidity (pH 5.2), and the high concentration of $MgCl_2$ in MSRV plates effectively reduces the growth of background microflora. Following point-inoculation of MSRV with preenrichment (direct MSRV) or selective enrichment cultures following preenrichment (indirect MSRV), overnight incubation of the inoculated plating medium at 42° C to 43° C favors the radial migration of motile salmonellae. Growth from the perimeter of the zone of migration is then tested with polyvalent antisera and/or separated on differential *Salmonella* plating media for subsequent biochemical and serological characterization.[191] A minimum of 48 hours (direct MSRV) or 72 hours (indirect MSRV) is therefore required to obtain presumptive serological results. The threshold sensitivity of direct MSRV is 60 salmonellae per ml of preenrichment culture.[188] Nonmotile *Salmonella* strains yield false-negative results,[193,194,547] whereas related microorganisms such as *Enterobacter cloacae* and the holding of incubated MSRV plates at room temperature for several hours can produce false-positive results.[484,691] Studies on the sensitivity of direct MSRV indicate that the approach compares favorably with standard methods of detection and that the MSRV is more reliable with low- than with high-moisture foods (Table 45–21). The observation that indirect MSRV is only marginally superior to direct MSRV may stem from the use of short (8 to 12

Table 45–21 MSRV Detection of *Salmonella* in Foods and Environmental Samples[a]

Sample	*Number of Samples* Tested	Positive[b]	*MSRV-Percent Positive** Direct[c]	Indirect[d]	Reference
High moisture					
Minced meat; egg yolk	53	15[e]	100	ND[f]	191
Coastal waters (marine)	256	83[e]	37.3	54.2[g]	506
Raw pork and chicken	116	69[e]	94.2	ND	194
Layer hen tissues (necropsy)	2,620	123[e]	77.2	ND	526
Raw poultry skin	NA**	91[e]	72.5	ND	193
Raw meats	1,393	321[h]	94.4	ND	516
Raw/processed meats	204	57[e]	NA	7.0	113
Chicken carcasses; giblets	235	61[e]	70.5	ND	632
Poultry litter/environmentals	209	104[e]	94.2	ND	547
Poultry/bovine tissues	1,387	480[e]	99.8	ND	185
Raw chicken/giblets; eggs	180	150[e]	22.0	ND	150
Raw poultry/beef/pork	419[i]	154[e]	87.0	ND	691
Low moisture					
Cocoa products; milk powder	395	61[e]	98.4	ND	191
Cocoa/chocolate products	450	441[h]	74.6	64.6[j]	192
Milk powder; environmentals	316	92[e]	96.7	ND	194
Cocoa/chocolate products	565	353[e,h]	91.2	81.0[j,k]	189
Milk powder; pasta; feeds	944	218[h]	97.7	ND	516
Chocolate; milk powder	52	22[e,h]	68.2	ND	183
Cocoa powder; chocolate	780	415[h]	93.3	94.2[k]	190
Animal feeds; environmentals	1,655	1,200[e]	96.2	ND	185
Milk powder; environmentals	210	100[e,h]	82.0	ND	369
Poultry feeds	1,345	125[e]	89.6	ND	488
Dried milk products	860	828[h]	96.9	97.5[j]	91

[a] MSRV usually incubated for 16 to 24 hours at 42° C to 43° C
[b] Sample positive (confirmed) by one or more analytical methods
[c] Preenrichment culture (16 to 24 hours) plated on MSRV
[d] Selective enrichment culture (6 to 8 hours) inoculated from preenrichment culture was plated on MSRV
[e] Naturally contaminated samples
[f] Not done
[g] Selenite F (35° C) and RV (43° C) broths incubated for 24 hours
[h] Artificially contaminated samples
[i] Includes 115 low-moisture products
[j] RV (42°C) incubated for 8 hours only
[k] SC (35° C) and/or TBG (35° C) incubated for 8 to 12 hours
* Proportion (%) of total number of positive samples
** Not available

h) periods of enrichment in selective broth media. The sensitivity of indirect MSRV increases significantly if longer (24 h) periods of enrichment are applied (D'Aoust, unpublished data). Of 100 naturally contaminated foods and agricultural materials, 88% were detected by direct MSRV, whereas 96%, 98%, and 91% were detected by indirect MSRV following 24 hours enrichment in TGB_{43}, TBG_{35}, and RV_{43}, respectively. Corresponding values for 6 h enrichment were 93%, 94%, and 81%, respectively.

In standard cultural procedures involving a preenrichment step, initiation of sample analysis is restricted to Mondays and Tuesdays if weekend work is to be avoided and if analyses are to be interrupted only by the refrigeration of incubated plating media. A novel refrigeration approach provides for the preenrichment of food samples on Mondays to Thursdays, inclusively. Following refrigerated (2° C to 10° C) storage of preenrichment and/or enrichment broth cultures over the weekend (72 h), the analysis of stored cultures is resumed on the next Monday. The sensitivity of the refrigeration approach varies with food groups. With high-moisture foods, the method detected, respectively, 88% to

95% and 91% to 100% of contaminated samples from the refrigerated preenrichment and enrichment cultures.[178,180] With low moisture foods, sensitivities of 99% to 100% and 99% to 100% were obtained.[171,178,180] The technique, which was recently recognized in standard methods,[14,168] is most valuable because it significantly increases laboratory flexibility and productivity.

45.8.2 Rapid Methods

Standard cultural procedures require 4 days to presumptively identify *Salmonella* in a test sample and up to 7 days to confirm the identity of the isolate.[165] This delays the release of foods and increases production costs. Efforts at greater method brevity have led to the commercialization of more than 30 diagnostic systems for the rapid detection of *Salmonella* spp. in food and agricultural products. These techniques insert in standard cultural methods at the level of preenrichment or after 6 hours (products with low numbers of background flora) or 24 hours (products with high numbers of background flora) of selective enrichment. Postenrichment cultures (18 and 6 hours) inoculated from 6 hours and 18 to 24 hours enrichment cultures, respectively, are used widely in rapid methodologies. Negative or presumptively-positive results are obtained 12 to 48 hours earlier than with conventional culture analyses.[165]

The detection of salmonellae by direct or indirect sandwich ELISA procedures hinges on the recognition of unique somatic (O) and/or flagellar (H) antigens by monoclonal or polyclonal antibodies (Table 45–22). ELISA procedures generally use microplates, strip wells, or dipsticks precoated with capture antibodies to specifically bind *Salmonella* antigens in test materials. The antigen-antibody complex then is revealed colorimetrically or fluorometrically by reaction with a specific peroxidase- or phosphatase-labeled conjugate and addition of enzyme substrate and chromogen. The threshold sensitivities of ELISA kits range from 10^4 to 10^6 salmonellae per ml of test culture with great disparity in kit specificity (Table 45–22). The strip-well Salmonella-Tek™ system (Organon Teknika, Durham, NC) relies on two monoclonal antibodies (MOPC 467 and 6H4), a peroxidase conjugate, and a H_2O_2/tetramethylbenzidine (TMB) chromogen system for the colorimetric detection of target antigens in heated M-broth postenrichment cultures. Validation studies using naturally and artificially contaminated foods and agricultural materials have reported false-negative rates of less than 6% and appreciably greater levels of false-positive reactions arising from the low specificity of the MOPC 467 antibody.[170] A major change in protocol in favor of enrichment at a high temperature (42° C) in the presence of novobiocin likely contributed to the enhanced specificity of the system in more recent studies.[165,217] Negative or presumptive-positive results are obtained within 50 to 56 hours following sample preparation. The manufacturer of Salmonella-Tek™ recently introduced the Capture-Tek™ format that detects salmonellae previously concentrated from preenrichment cultures by immunomagnetic separation using Dynal Dynabeads™ and subsequent enrichment of Dynabeads™ with adsorbed salmonellae in M broth supplemented with novobiocin for 5 hours at 42° C. The performance of this technique, which yields negative or presumptive-positive results in less than 30 h, has yet to be fully validated. In the Tecra™ visual immunoassay (Bioenterprises PTY Ltd, Roseville, NSW, Australia), *Salmonella* somatic and flagellar antigens in heated M-broth postenrichment cultures are captured on the surface of antibody coated strip- or microplate wells. The immune complex then is reacted with a peroxidase conjugate and revealed by the addition of enzyme substrate and azino-benzthiazoline sulfonate chromogen. Mean false-negative and false-positive rates of 3.8% and 7.3% (Table 45–22) with frequent identifications of *Escherichia coli*, *Citrobacter*, and *Enterobacter* as sources of false-positive reactions have been reported.[152,357] More recently, the Unique™ dipstick module was introduced by Bioenterprises PTY Ltd for the determination of salmonellae in overnight preenrichment cultures. A dipstick coated with capture antibody is moved sequentially through a six-compartment module, thereby providing for the immunoenrichment of the target organism in M-broth and its presumptive colorimetric identification from reactions of the capture antibody-antigen complex on the dipstick with an alkaline phosphatase conjugate and bromo-chloro-indoyl phosphate (BCIP) substrate. The system, which provides presumptive-positive results within 22 h of sample preparation, yielded false-negative and false-positive rates of less than 6.0% and 9.0%, respectively (Table 45–22). The Locate™ system (Rhône Poulenc Diagnostic Ltd., Glasgow, Scotland) is a microplate direct ELISA procedure that relies on monoclonal antibodies for the detection of somatic antigens in heated GN postenrichment broth cultures. Color development follows from the reaction of a peroxidase conjugate with substrate and TMB chromogen. Sample analysis is completed within 50 to 56 hours and the method provides good sensitivity and specificity (Table 45–22). The Equate™ direct microplate ELISA (Binax, South Portland, ME) requires less than 48 hours for the detection of *Salmonella* antigens in acid-treated M-broth postenrichment cultures. Immune complexes from the microwell reaction of precoated monoclonal antibodies and peroxidase conjugate with target antigens are revealed by the addition of enzyme substrate and TMB chromogen. Isolated reports on the performance of this system suggest good sensitivity but low specificity (Table 45–22). The microplate indirect Assurance™ assay (BioControl Systems Inc., Bothell, WA) uses proprietary polyclonal antibodies for the detection of *Salmonella* antigens in heated M-broth postenrichment cultures. Test configuration includes reaction of the immune complex consisting of targeted somatic (O) and/or flagellar (H) antigens and surface coated antibody with a second *Salmonella*-specific polyclonal antibody termed Assur-Link.™ The latter antibody minimizes stearic hindrance during the binding of the large enzyme conjugate polymer to the targeted microwell immune complex.[235] The capture antibody-antigen-Assur-Link™ complex is then reacted with a third polyclonal antibody-alkaline phosphatase conjugate followed by color development using a p-NO_2 phenyl phosphate substrate. Negative or presumptive-positive results are obtained within 50 hours of sample preparation. The Assurance™ assay yields low rates of false-negative and false-positive reactions (Table 45–22). The Vitek Immunological Diagnostic Assay System (Vidas™, bioMérieux sa, Marcy-l'Etoile, France) is an automated immunofluorometric system for the detection of *Salmonella* somatic (O) and flagellar (H) antigens in heated M-broth postenrichment cultures. This direct ELISA procedure relies on monoclonal (capture) and polyclonal (conjugate) antibodies for detection. One of the two principal components of the Vidas™ system is a solid phase receptable (SPR), a solid matrix in the shape of a pipette tip whose internal surface is precoated with capture antibody. The other component is a ready-to-use reagent strip that contains all the necessary reagents for in-line processing of the test sample. The reagent strip previously inoculated with heated M-broth culture and its corresponding SPR are seated in the instrument. The SPR is displaced successively into each of the 10 reagent chambers where reagents are automatically and repeatedly cycled into and out of the SPR. The intensity of blue fluorescence produced in the last chamber from the reaction of the conjugated alkaline phosphatase with methyl-umbelliferyl phosphate is then measured. The immunofluorescent assay including instrument interpretation of fluorometric results requires 40 to 50 minutes, whereas the entire test protocol is completed within 50 hours of sample preparation. Performance assessments of the Vidas™ system have yielded favorable results except for an unusually high level of false-negative reactions in one study that likely resulted from the use of short (6 hours) enrichment in RV_{42} and an inappropriate 1:10 preenrichment to enrichment transfer volume in the analysis of raw poultry samples.[151] *Citrobacter freundii* can produce false-positive Vidas™ reactions.[85]

Bacterial motility is used as a diagnostic tool in several technologies (Table 45–23). In the immunoimmobilization 1-2 Test™ (BioControl Systems Inc., Bothell, WA), motile salmonellae in selective enrichment cultures are presumptively identified within 50 hours of sample preparation. The assay hinges on the formation of a white precipitin band at the interface of salmonellae in a semi-solid medium and polyvalent flagellar (H) antisera diffusing toward the migrating target microorganism. The requirement for a single-reaction vial per test sample is cost effective because it prevents the waste of unused reagents as in precoated microwell ELISA. False-negative and false-positive rates of up to 10.6% and 5.1% have been reported in studies involving naturally and artificially contaminated test products (Table 45–23). The use of only one selective enrichment medium in the 1-2 Test™ protocol may limit test sensitivity particularly with products containing high levels of background microflora. *Citrobacter freundii, Enterobacter cloacae,* and *Klebsiella pneumoniae* may react in this assay.[679] The Oxoid Salmonella Rapid Test (Unipath Limited, Basingstoke, UK) is a 43-h test procedure that presumptively identifies motile salmonellae in preenrichment cultures using discriminant biochemical reactions. The system consists of two tubes each separated into an upper and lower chamber by a porous partition. The lower chamber of each tube contains different selective enrichment broth media. The upper chamber of one tube detects H_2S gas formation, whereas the upper chamber of the second tube monitors H_2S formation and acid production from lactose. Observation of such reactions in either or both upper chambers is indicative of the presence of salmonellae. Clearly neither of these systems will detect nonmotile *Salmonella* and the occurrence of atypical biotypes may have affected the performance of the Oxoid test system (Table 45–23).

Table 45–22 Performance of Commercial ELISA Systems for the Detection of *Salmonella*

		Number of Samples		*Percent Erroneous Results*		
System	*Product*	*Tested*	*Positive*[a]	*False (−)*[b]	*False (+)*[c]	*Reference*
Salmonella-Tek	Raw poultry, dry foods	1,000[e]	595	1.7	2.0	217
	Raw poultry, feeds, dry foods	2,350[d,e,f]	1,680	2.5	20.6	165
	Feeds	225[d,e]	125	5.6	14.0	676
Tecra microplate	Raw poultry, feeds, dry foods	1,971[d,e,f]	982	3.8	7.3	165
Locate	Sewage waters	180[d]	66	0.0	5.3	678
	Raw poultry, dry foods	1,439[d,e]	789	1.3	3.5	252
Unique	Raw meats, spices, egg products	20[e]	20	0.0	0.0	367
	Meats, feeds	50[d]	17	5.9	9.1	638
	Raw poultry, environment	100[d]	60	1.7	1.6	524
Equate	Raw meats	120[d]	17	0.0	12.6	222
	Raw meats, feeds, dry foods	96[d,e]	47	4.3	12.2	327
Assurance	Raw poultry, dry foods	1,049[d,e]	546	1.8	3.6	234
	Raw meats, feeds, dry foods	1,000[d,e]	722	1.8	0.0	235
Vidas	Raw meats, dairy, dry foods	141[d,e]	83	4.8	6.9	85
	Raw poultry, dry foods	1,544[d,e]	793	1.3	2.1	151
	Raw poultry	151[d]	76	1.3	0.0	503

[a] Culturally confirmed
[b] Percent ratio of false-negative results to total number of confirmed positive samples
[c] Percent ratio of false-positive results to total number of true negative samples
[d] Naturally contaminated
[e] Artificially contaminated
[f] Results of different studies were combined

The application of conductimetric, DNA hybridization, and immunodiffusion principles has resulted in innovative techniques for the rapid detection of foodborne salmonellae. The Malthus™ conductimetric assay presumptively identifies *Salmonella* in preenrichment cultures within 54 hours of sample preparation using paired conductance liquid media (SS_1 and SS_2). This automated system measures the change in electrical conductance associated with increasing concentrations of ionic species attributed to *Salmonella*-specific acid production from dulcitol and reductase-dependent conversion of trimethylamine oxide to trimethylamine (SS_1), and production of cadaverine from the decarboxylation of lysine (SS_2). Although SS_1 and SS_2 contain SC as selective agent, both media can give false-positive reactions with *Citrobacter* and *Hafnia alvei*, respectively.[214,487] High false-negative reaction rates and low specificity have also been reported (Table 45–24). Preliminary results with novel conductance media (M_1 and M_3) of enhanced selectivity from the addition of antibiotics and incubation at elevated temperature are encouraging.[92] Several DNA-based methods have been developed for the detection of bacterial pathogens, including the GENE-TRAK™ (GENE-TRAK Systems, Framingham, MA) assay for foodborne *Salmonella*. The system presumptively identifies salmonellae in Gram-negative postenrichment cultures within 50 hours of sample preparation. GENE-TRAK™ relies on synthetic capture and detector oligonucleotide DNA probes that hybridize to proximal but different *Salmonella*-specific ribosomal RNA sequences. The detector probe consists of a 35 to 40 nucleotide sequence labeled at both the 3' and 5' ends with fluorescein, whereas the capture probe of approximately 30 nucleotides contains a polydeoxyadenylic (poly-dA) tail attached to the 3' end of the probe sequence. A dipstick coated with a homopolymer of thymidine residues (poly-dT) is used to retrieve the [capture probe-rRNA-detector probe] complex from the reaction mixture. The complex then is reacted with a polyclonal antifluorescein antibody conjugated to horseradish peroxidase followed by color development using H_2O_2 and TMB chromogen. The combined results of eight validation studies showed mean rates of false-negative and false-positive reactions of 2.0% and 1.6%, respectively (Table 45–24). The reasons for the unusually high rate of false-negative reactions with artificially contaminated feeds are uncertain.[676] To fur-

Table 45–23 Performance of Commercial Motility Systems for the Detection of *Salmonella*

		Number of Samples		*Percent Erroneous Results*		
System	*Product*	*Tested*	*Positive*[a]	*False (−)*[b]	*False (+)*[c]	*Reference*
1-2 Test	Raw meats, feeds, dry foods	1,250[d]	437	5.0	1.4	681
	Raw poultry, feeds, dry foods	2,155[d,e,f]	1,025	4.3	3.3	165
	Raw meats, feeds	760[d,e]	371	7.0	5.1	679
	Complete feeds	1061[d]	85	10.6	1.1	488
	Raw meats, feeds	320[d,e]	215	2.8	2.9	233
	Dry foods, feeds	550[d,e]	349	0.0	0.0	236
Oxoid Rapid Test	Raw meats, feeds, dry foods	820[d,e]	223	3.1	4.4	328
	Raw meats, feeds, dry foods	96[d,e]	47	2.1	0.0	327
	Cattle feces	100[d]	19	5.3	2.5	684
	Raw chicken	77[d]	33	13.3	6.4	322
	Raw meats, dry foods	120[e]	103	22.3	NS[g]	347

[a] Culturally confirmed
[b] Percent ratio of false-negative results to total number of confirmed positive samples
[c] Percent ratio of false-positive results to total number of true negative samples
[d] Naturally contaminated
[e] Artificially contaminated
[f] Results of different studies were combined
[g] Not stated

ther simplify its analytical protocol, GENE-TRAK™ recently altered the configuration of the detector probe by replacing the fluorescein label and antifluorescein peroxidase conjugate with a direct labeling of the detector probe with peroxidase. The Path-Stik™ (Lumac B.V., Landgraaf, The Netherlands) is an immunochromatographic technique for the presumptive identification of salmonellae in BPW postenrichment cultures within 50 h of sample preparation. The appearance of a blue line in the test area of the Path-Stik™ dipstick results from the capillary comigration and reaction of target antigen with colored latex particles coated with *Salmonella*-specific antibody and capture of the migrating antigen by a second *Salmonella*-specific antibody immobilized in the test area. Results with the Path-Stik™ test (Table 45–24) clearly indicate the need for improved sensitivity possibly through greater selectivity of the enrichment protocol and enhanced antibody affinity for *Salmonella* antigens.

Several novel technologies are available whose performance has yet to be fully evaluated. The Bacterial Ice Nucleation Detection (BIND™) system (Idetek Inc., Sunnyvale, California) is a microplate colorimetric assay for the detection of salmonellae in overnight preenrichment or direct enrichment cultures. The system, which yields negative or presumptive-positive results in less than 24 hours, is based on a *Salmonella*-specific phage transduction of an ice nucleation activity (*ina*) gene that encodes a template protein for the formation of ice crystals.[697] The transduction and synthesis of nucleation proteins by *ina*$^+$ salmonellae are detected by the microwell freezing (−9.3° C) of a carboxyfluorescein dye (orange-red), whereas no change in the green fluorescent dye confirms the absence of *Salmonella* in the test sample. Preliminary results from the manufacturer indicate a threshold sensitivity of ≥200 salmonellae per milliliter and high specificity of the BIND™ technique. The polymerase chain reaction (PCR) is a sensitive and specific technique for the amplification of a target sequence of nucleic acid.[302] The procedure generally involves alternate cycles of heat denaturation of double-stranded DNA into single strands, annealing of oligonucleotide primers to a region in the single-stranded DNA that flanks the targeted sequence, and use of a thermostable *Taq* polymerase to replicate the targeted sequence. Potential problems with this technology include inhibition of the polymerase reaction by food components, positive PCR responses from nonviable target microorganisms, and environmental cross-contamination of test samples or pre-amplification reagents. Interest in the application of PCR technol-

Table 45–24 Performance of Other Commercial Systems for the Detection of *Salmonella*

System	Product	*Number of Samples*		*Percent Erroneous Results*		*Reference*
		Tested	*Positive*[a]	*False (–)*[b]	*False (+)*[c]	
Malthus conductance	Raw meats, feeds, dry foods	1,280[d]	566	11.1	0.7	271
	Raw meats, pasta, cheese	111[e]	10	10.0	13.9	714
	Dairy plant environment	210[d,e]	100	34.0	62.7	369
GENE-TRAK	Raw poultry, feeds, dry foods	4,238[d,e,f]	2,593	2.0	1.6	165
	Feeds	32[d]	22	36.4	0.0	676
Path-Stik	Raw meats, dry foods	166[e]	29	31.0	5.8	366
	Raw meats, egg products, dry foods	94[e]	40	7.5	3.7	100
	Raw poultry	48[e]	26	61.5	0.0	503

[a] Culturally confirmed
[b] Percent ratio of false-negative results to total number of confirmed positive samples.
[c] Percent ratio of false-positive results to total number of true negative samples
[d] Artificially contaminated
[e] Naturally contaminated
[f] Results of different studies were combined

ogy (Chapter 64) already has produced three commercial diagnostic products for the detection of foodborne *Salmonella*. The BAX™ system (DuPont Company, Wilmington, Delaware) provides presumptive evidence of salmonellae in overnight preenrichment cultures within 30 h of sample preparation. Following the separation of PCR products by gel electrophoresis, the identity of *Salmonella*-specific amplicons is confirmed by comparison with electrophoretic control bands. Preliminary results (May 1996) from a study by Campden and Chorleywood (Chipping Campden, UK) indicated a threshold sensitivity of 10^2 to 10^3 salmonellae ml^{-1} of pure culture and high levels of sensitivity in the detection of *Salmonella* in inoculated and naturally contaminated foods. The Probelia™ system (Sanofi Diagnostics Pasteur, Marnes La Coquette, France) is a colorimetric PCR technique for the presumptive identification of *Salmonella* in overnight preenrichment cultures. The *iagA* gene within the *Salmonella* invasion apparatus is amplified, and PCR products are captured in microwells precoated with a specific probe. The resulting probe-PCR product complex is revealed by reaction with a amplicon-specific peroxidase-labelled probe and addition of enzyme substrate and TMB chromogen. Preliminary results from the manufacturer underline the unfailing sensitivity and specificity of the Probelia™ technique. The TaqMan™ system (Perkin-Elmer Biosystems, Foster City, California) is an automated fluorometric procedure for the determination of salmonellae in preenrichment cultures within 24 hours of sample preparation. PCR amplification of the targeted *invA* gene is detected by a fluorogenic probe covalently labelled with a 5'-reporter and a 3'-quencher dye. The proximity of the reporter and quencher dyes in the intact probe suppresses fluorescence, whereas enzymic cleavage of the fluorogenic probe results in increased fluorescence. In the TaqMan™ assay, primers hybridize to the targeted *invA* sequence, which is then amplified by the AmpliTaq DNA polymerase in a 5' to 3' replication process. During amplification, the fluorogenic probe binds to an alternate target sequence within the generated PCR fragment but is subsequently hydrolyzed by the 5'-nuclease activity of the AmpliTaq DNA polymerase, thereby releasing the reporter dye and increasing the fluorescence signal. Pure culture studies conducted by the manufacturer indicate a product sensitivity and specificity of 99.7% and 100%, respectively.

The last decade has witnessed the development of numerous methods for the rapid detection of foodborne *Salmonella*. Although ELISA kits (Table 45–22) readily detect salmonellae belonging to somatic groups B to E, the ability of these kits to detect exotic serovars that are found increasingly in foods imported from Third World countries has yet to be ascertained. Moreover, foodborne *Citrobacter, Enterobacter,* and *Escherichia* may yield false-positive reactions. The uncertainty surrounding the current and future prevalence of nonmotile salmonellae and the impact of environmental conditions on the synthesis of functional flagella detracts from methods that depend on bacterial motility (Table 45–23). The reliance of PCR and probe (Table 45–24) technologies on stable genomic loci augurs favorably for the future development of sensitive, specific, and cost-effective methodologies for the detection of foodborne *Salmonella*.

45.9 CONTROL

The ubiquity of *Salmonella* in the natural environment and the disquieting increased incidence of antibiotic-resistant salmonellae emphasize the public health importance of stringent controls on the production, processing, and distribution of foods and food ingredients. The control of *Salmonella* in meat animals and meat products presents a formidable challenge because of the complex organization of the meat industry and numerous opportunities for product contamination within the farm to retail continuum.[703] The intensive husbandry practices used in the bovine, porcine, and poultry meat industries promote intestinal carriage and surface contamination of live animals.[160] The problem is likely to be further compounded by the spread of untreated sludge and animal waste on farmlands, which contaminates surface waters and exposes grazing herds to infection. Stress-induced shedding of *Salmonella* as a result of food or water deprivation, prolonged holding in lairage pens, induced molting, and transport of live animals to slaughtering plants further increase the surface contamination of meat animals.[316,468,469] The inability to consistently produce *Salmonella*-free replacement stocks and animal feeds (Table 45–6) results in a pyramidal increase in animal infection and exacerbates control efforts within the red meat and poultry industries.[160,690] For example, the introduction of day-old chicks from an infected multiplier breeder flock in freshly sanitized poultry houses and provision of contaminated feeds would likely negate the anticipated benefits of Hazard Analysis Critical Control Point (HACCP) control measures and good farming practices, such as disinfestation and biosecurity of rearing premises, controlled access to poultry houses, provision of clean drinking water, and control of airborne contamination. Improvements in the bacterial quality of rendered meat products depend on strengthening of in-plant sanitary practices and process controls, replacement of aging equipment with more efficient units that can be easily dismantled and sanitized, physical isolation of raw meat processing areas, and protection of thermally processed materials from recontamination.[160,186] Although conventional steam conditioning of feeds at 82° C or greater prior to pelleting constitutes a positive step toward *Salmonella*-free feeds, recontamination of finished products during feed mill operations, bulk storage, transportation, and on-farm storage needs to be more effectively controlled.[143,319,703] The greater agricultural use of feeds treated with Bio-Add™ (Trouw UK Ltd, Norwich, UK) or Sal Curb™ (Kemin Europa N.V., Herentals, Belgium) mixtures of formic and propionic acids or with other nonantibiotic additives to inhibit or inactivate secondary contaminants would improve product safety.[73]

Vaccines are an important on-farm measure to combat *Salmonella* in animal husbandry.[160,703] Parenteral live or killed vaccines for protection against *S.* Gallinarum (poultry), S. Enteritidis (poultry), S. Dublin (bovine), *S.* Choleraesuis (porcine), and *S.* Typhimurium, which indiscriminately infects farm animals, are available. These animal vaccines afford little or no cross-protection against other *Salmonella* serovars. The use of genetically defective mutants ensures that live vaccines are avirulent.[114,138,674] Competitive exclusion of salmonellae in layer and broiler flocks is based on the ability of orally administered cultures prepared from the fecal or cecal microflora of *Salmonella*-free adult birds to protect chicks against *Salmonella* intestinal colonization.[622] The unknown composition of these probiotic mixtures of facultatively and strictly anaerobic microorganisms poses major problems in terms of vaccine standardization and raises concerns about the possible presence of avian pathogens. The protective capacity of these mixes largely has been assessed in laboratory scale experiments involving the oral treatment of day-old chicks with the protective mixture and oral challenge of treated birds with *Salmonella* on the following day. The efficacy of treatment is assessed in terms of the prevalence of *Salmonella* in necropsied tissues of control and treated birds euthanized 10 or more days following challenge. Several field studies in Europe and North America indicate that the use of undefined mixtures coupled with good hygienic practices on poultry farms do not consistently reduce *Salmonella* in commercial poultry flocks.[622] Undefined mixtures marketed under the trade names of Broilact™ (Orion Corporation Farmos, Turku, Finland) and Aviguard™ (Life-Care Products Limited, Malvern Link, England) are currently available.[90,622] Attempts to develop defined mixtures that effectively protect breeder and broiler birds against *Salmonella* infec-

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tion have met with limited success.[622] The addition of vermicompost, an earthworm-dependent digest of adult chicken feces, to the feed of newly hatched chicks afforded protection against *Salmonella* colonization.[618]

Many operations in bovine and porcine slaughterhouses contribute to carcass contamination.[76,93,201,703] The removal of animal hides and fleece soiled with fecal material and the scalding, dehairing, and polishing of pork carcasses greatly favor cross-contamination of meat surfaces.[201,272,703] In addition to the use of a steam vacuum to dislodge and aspirate feces and ingesta from hide surfaces,[41] thermal or chemical decontamination of scald water prior to its recycled use in the dehairing process[272] would enhance meat quality and safety. Sanitizing meat carcasses by immersion or spray-washing with cold and hot water, chlorine, chlorine dioxide, quaternary ammonium compounds, trisodium phosphate (AvGard™), ozone, hydrogen peroxide, and organic acids is well documented.[155,201,202,283,532,533] Hot (74° C) water,[283] hot (65° C to 80° C) chlorinated water,[8] and hot (53° C to 55° C) dilute acetic or lactic acid spray-washing of carcasses are gaining favor over the more traditional temperate water rinsing of eviscerated meat carcasses.[201,532] Spray decontamination of carcasses is preferable to immersion because the full potency of bactericidal agents is retained and not reduced by the suspended organic debris in wash-water tanks. Innovative equipment for the steam pasteurization of eviscerated beef carcasses gradually is being introduced into North American slaughterhouses.[40] The merits of other sanitizing technologies such as gamma and electron irradiation, electromagnetic waves, and pulsed electric or magnetic fields have also been considered.[141]

The hygienic slaughter of poultry is difficult because of the intestinal carriage of *Salmonella* in asymptomatic birds and surface fecal contamination of birds arriving at the abattoir. In conjunction with common feed withdrawal practices,[703] the use of properly sanitized transport crates,[218,559] and reduction in the number of birds per crate to reduce shipment stress would markedly reduce contamination of bird surfaces. Commercial scalding at 52° C to 60° C for up to 2 min and the defeathering of bird carcasses constitute weak links in the hygienic processing of poultry. The serial passage of fecally soiled birds in scald tanks and limited replacement of contaminated scald waters result in widespread cross-contamination of birds. Immersion scalding at temperatures exceeding 60° C is contraindicated because it damages the protective (hydrophobic) keratinized epidermal layer of the poultry skin and enhances the attachment of salmonellae to underlying tissues.[134,387,611] A similar condition prevails in the kosher process where cold (7° C) scalding necessitates a lengthy defeathering process that damages poultry skin and encourages bacterial attachment.[134,385] Acidification of scald water with organic acids yields carcasses that are unacceptable organoleptically.[421] Immersion tank scalding at pH values of 9.0 to 11.0, scalding in tanks operated under counter-current flow, successive scalding in three immersion tanks regularly replenished with fresh water, steam scalding of birds on the conveyor line, simultaneous scalding and plucking using a hot (62° C) water spray and final washing of scalded birds with chlorinated water, cetylpyridinium chloride, and other bactericidal agents have been advocated for greater reduction of *Salmonella* on poultry carcasses.[38,160,340,386,470] Following defeathering and evisceration, carcasses are cooled in counter-flow immersion chill tanks (spin chillers) or in a stream of cold air.[160,419] Other possible chill technologies of interest include cryogenic chilling with liquid nitrogen or solid carbon dioxide and evaporative chilling resulting from the vacuum evaporation of moisture on warm carcasses.[419] Comingling of large numbers of carcasses in spin chillers contributes to cross-contamination, which depends greatly on the level of salmonellae on eviscerated birds entering the spin chiller.[419] Several bactericides including chlorine, chlorine dioxide, organic acids, propylene glycol, and ozonation of recycled chill waters have been proposed for the reduction of *Salmonella* in chill water.[354,418,419,642] The ability of the AvGard™ trisodium phosphate wash solution to decontaminate carcasses and to effect a 2.0 $\log_{10}$ or greater reduction in surface *Salmonella* contamination putatively stems from its highly alkaline pH rather than a cleansing action of the inorganic salt.[139,420,708] Seemingly, the alternate use of NaOH to attain high pH values would be equally effective without raising environmental concerns on the disposal of insoluble phosphates in spent AvGard™ solutions.

Effective intervention strategies in the shell egg industry are needed to reduce the ongoing human pandemic of *S.* Enteritidis infections. Control measures include *Salmonella*-free replacement layer birds, probiotic and vaccine prophylaxis, *Salmonella*-free feeds with added nonantibiotic preservatives, and stringent biosecurity in layer farm operations. In addition to the traditional washing of shell egg surfaces in warm water adjusted to pH 10.0 or greater,[329,464] other bactericidal wash treatments using chlorine, organic acids, trisodium phosphate, iodophore, or H_2O_2, as well as ultraviolet irradiation have been considered for surface decontamination.[284,336,397,398,464] Prompt refrigeration of graded shell eggs and their cold storage (7° C or lower) during distribution and retail display would retard the growth of internalized salmonellae. The diversion for pasteurization of shell eggs from layer flocks infected with *S.* Enteritidis would improve public health safety.[11,447] A recently marketed in-shell pasteurization system that reportedly effects a 5.0 $\log_{10}$ reduction of salmonellae in egg magma from the immersion of intact shell eggs in a water bath held at 56° C (Pasteurized L.P.™, Laconia, NH) is most promising. A similar lethality was reported for shell eggs held in a hot air oven (55° C) for 3 hours.[336]

The growing importance of fresh fruits and vegetables as infectious vehicles of *Salmonella* and other bacterial and parasitic disease agents warrants a strengthening of hygienic practices in the production, distribution, and marketing of these ready-to-eat products. The agricultural use of clean irrigation water, treatment of animal wastes before use as fertilizer, and education of field workers in personal hygiene and hygienic handling of fresh produce would greatly alleviate the health risk associated with these products.[96,345] Postharvest wash waters containing chlorine, ClO_2, organic acids, trisodium phosphate, H_2O_2, and surface coating of fresh produce with a shellac-based water wax reportedly reduce surface contamination of fruits and vegetables.[499,585,685,715,717] Cold shipment of produce in refrigerated vehicles or on ice made from hyperchlorinated water is highly recommended. Decontamination of sprouting seeds requires prolonged soaking in chlorinated water, calcium or sodium hypochlorite, H_2O_2 or ethanol, or the heating of seeds in a water bath for 5 minutes at 57° C.[82,356] Modified or controlled atmosphere packaging extends product shelf-life by replacing air with high concentrations of CO_2 mixed with N_2 and/or O_2 to inhibit the growth of aerobic spoilage microorganisms.[162,335] The approach neither promotes the growth nor adversely affects the survival of *Salmonella*.[162] Bacteriostasis is also favored by the cold storage of packaged fruits and vegetables at low relative humidity. Consumers and handlers in food service and retail food establishments should be aware of the health hazards from the unrefrigerated storage of cut produce, which exposes plant tissues to bacterial invasion and proliferation.[49,226,280,422,716]

Aquaculture is a relatively new food industry where six of the nine major producers operate in Asia, led by the People's Republic of China with an approximate output of 4.0 × 10^6 metric tons in 1985.[625] The prevailing hygienic conditions in these countries potentiate the contamination of unprotected rearing ponds and aquacultural products with human pathogenic microorganisms.[163,164] Feeding of reared species with raw meat scraps, animal feed, poultry manure, and human excrement exacerbates public health risks.[423,550,551] These concerns are compounded by the widespread on-farm use of antimicrobials where accumulation of excess drugs in pond sediments selects for antibiotic-resistant bacteria.[50,103,164] The occurrence of resistant strains in cultured fish attests to the hazard of this aquacultural practice.[196,307,582,657] Moreover, a growing industrial application of the newer quinolones and fluoroquinolones for therapy and prophylaxis is disquieting because it favors the emergence of bacterial pathogens that exhibit cross-resistance to medically important fluoroquinolones. The spectre of bacterial quinolone-resistance in aquacultural products that frequently are cooked lightly prior to consumption reinforces the need for more stringent hygienic controls and a ban on the prophylactic and therapeutic use of fluoroquinolones in aquacultural operations in developed and in Third World countries. The increasing use of antimicrobials on fruit and vegetable crops is yet another public health concern, although the impact of this horticultural practice has yet to be assessed.

Government endorsement of irradiation for the reduction of spoilage microflora and the elimination of human bacterial pathogens in foods and food ingredients has provided the industry with a technology that promotes greater food safety and shelf stability (Chapter 4).[147,467] Irradiation exerts little or no adverse effects on the nutritional value and wholesomeness of irradiated foods.[273] From the early application of gamma irradiation to disinfest and inhibit sprouting of vegetable crops, numerous studies have confirmed the efficacy of radiation doses greater than 3.0 kGy to eliminate *Salmonella* in foods.[130,204,479,641] However, irradiation does not effectively destroy preformed bacterial toxins. In spite of conclusive evidence and assurances from the World Health Organization that low to medium doses of irradiation do not adulterate foods with potentially harmful radiolytic additives,[147] consumer apprehension on the safety of this bactericidal treatment remains an important hurdle in the successful marketing of irradiated foods.[535]

The continued prominence of human foodborne salmonellosis and clear evidence that this major health problem is increasing globally reiterates the need for concerted control efforts in all sectors of the food chain. The urgency of the situation is fueled by the pandemic spread and persistence of highly virulent strains such as S. Enteritidis and S. Typhimurium DT 104, the identification of new infectious food vehicles, and an alarming rise in foodborne *Salmonella* exhibiting multiple resistance to medically important antimicrobial drugs. Improvements in meat animal husbandry through the on-farm placement of noninfected stocks and provision of *Salmonella*-free feeds strengthened by improved hygienic slaughter of meat animals are essential. Efforts of the food industry to improve the quality and safety of food products continue to be undermined by the hazardous preferences of some consumers for raw milk cheese (Table 45–17), certified raw fluid milk,[529,637,686] raw fish (sushi) and bivalves, and raw minced beef crowned with raw eggs (steak tartare) that clearly defy logic, social responsibility, and self-preservation. Consumer education on the safe handling and cooking of foods remains a formidable challenge for the next century.

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46 *Shigella* species

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46.1 INTRODUCTION

Shigellosis, also known as bacillary dysentery, is an illness characterized by watery diarrhea, which is followed within a few days by the appearance of mucus in the stool, with or without blood. In 1898, a bacterium was isolated and characterized from a patient with bacillary dysentery by the Japanese bacteriologist Kiyoshi Shiga.[96] A few years later, work by Flexner[24] led to the isolation of a serologically related bacterium in the stools of patients with dysentery. Conradi[15] and Neisser and Shiga[72] indicated that a toxin (subsequently named Shiga toxin) may be responsible for pathogenesis. In 1950, the bacillus isolated by Shiga, *Bacillus dysenteriae*, was renamed *Shigella dysenteriae*.

Shigellosis is a diarrheal disease that affects millions of people yearly, not only in developing countries but also in industrial countries. Rapid spread of the disease can occur in crowded living quarters throughout the world, particularly in countries lacking the infrastructure to maintain proper sanitation. In developed countries, changes in lifestyle have led to increased numbers of day-care centers and nursing homes. Individuals in these facilities are at increased risk from the rapid dissemination of *Shigella*. Interestingly, diarrheal diseases, including those caused by *Shigella*, are sometimes a significant factor in military campaigns. In Saudia Arabia in 1991, 57% of United States (US) troops experienced diarrhea; the pathogens most frequently isolated from stool samples (432) were enterotoxigenic *Escherichia coli* (30%) and *Shigella* (26%).[40] Shigellosis can affect large segments of civilian populations by events such as civil war and the displacement of people into refugee camps as in Rwanda.[78] In 1993, an outbreak of bacillary dysentery in Mozambique affected 47,483 individuals, with 199 fatalities reported. *S. dysenteriae* type 1, apparently the leading cause of shigellosis in Africa, was identified as the pathogen responsible for this epidemic.[3]

Foodborne outbreaks of shigellosis occur worldwide and, in some instances, affect more than one country. A recent outbreak in several European countries was caused by consumption of contaminated lettuce imported from Spain.[27,47] Research into methods for the identification of sources of foodborne outbreaks by *Shigella* lag behind the technology developed for the clinical laboratory. Foods

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potentially at risk are not routinely screened for *Shigella* because this pathogen is spread principally by person-to-person contact and is not associated with any specific food.

LaBrec[54] demonstrated in 1964 that shigellae have the ability to invade epithelial cells; this was established as an essential step in the pathogenesis of bacillary dysentery. An important observation in elucidating the molecular events of *Shigella* pathogenesis was the correlation of the presence of a 180 to 220 kilobase pair (kbp) plasmid and the ability of *Shigella* to invade colonic epithelial cells.[87,88] Many of the plasmidborne genes responsible for pathogenesis of *Shigella* have been identified, sequenced, and characterized. The exact nature of the way in which these genes and the proteins they encode interact with the host environment and with each other has not been elucidated fully, although some models have been proposed.[126]

46.2 TAXONOMY AND CHARACTERISTICS OF THE ORGANISM

The genus *Shigella* contains four species that are Gram-negative, rod-shaped bacteria; it is taxonomically grouped in the family Enterobacteriaceae. The four species can be differentiated from each other by the fermentation of sugars, sugar alcohols, production of indole, and the synthesis of ornithine decarboxylase or arginine dehydrolase. Although these organisms are reported to be nonmotile, in a recent report, all four *Shigella* spp. (virulent and avirulent strains) were shown not only to possess flagella but also to be motile in low-percentage soft motility agar.[30] Members of the genus *Shigella* are genetically, biochemically, and serologically similar to *E. coli.*[12] One major difference between the two is the inability of *Shigella* to ferment lactose (although some do ferment lactose slowly). Most *E. coli* are lactose fermenters although enteroinvasive *E. coli* (*EIEC*), a causative agent of bacillary dysentery, is also a lactose nonfermenter. *Shigella* spp. are unable to produce gas during the fermentation of carbohydrates and cannot use citrate as a sole source of carbon.[13] Table 46–1 summarizes some of the biochemical tests used to differentiate *Shigella* from *E. coli.*

EIEC share many similarities with *Shigella*, ie, they contain the large virulence plasmid necessary for pathogenesis and possess an O-antigen, such as *EIEC* O124, that cross-reacts with several serotypes of *Shigella*. In this case, the identification of the causative agent in a foodborne disease incident can be difficult. For example, in an outbreak caused by *EIEC*-contaminated soft cheese, the initial identification was misdiagnosed as *Shigella.*[112] A significant difference between *EIEC* and *Shigella* is the infective dose; in *EIEC*, it is 10^6 bacteria and for shigellae, it is only 10 to 10^2 organisms.[19] The molecular events that are responsible for the difference in infectious doses are not known.

Table 46–1 Biochemical Reactions To Distinguish *Shigella* spp. from *E. coli*

	Result	
Test	Shigella	E. coli
Motility	–	+
D-glucose gas production	–*	+
Lactose acid production	–†	+
Mucate fermentation	–	+
Lysine decarboxylase	–	+
Christensen citrate	–	D
Acetate	–	+

* Strains of *S. flexneri* serovar 6 and *S. boydii* serovar 13 and 14 are positive.

† Strains of *S. flexneri* serovar 2a, *S. boydii* serovar 9 are positive, and *S. sonnei* are positive after a few days.

D: differrent reactions

Bacillary dysentery is caused by all four members of the genus *Shigella*, which are grouped serologically as follows: *S. dysenteriae* (group A), *S. flexneri* (group B), *S. boydii* (group C), and *S. sonnei* (group D) (Table 46–2). Groups A through C are further divided into more than 45 serotypes and subtypes based upon the O-antigens of the lipopolysaccharide (LPS). *S. sonnei* is the only group that has a single serotype. The range of severity of illness elicited by the various species correlates with their serogroup and a correlation seems to exist between serotype and geographical location.[52] Thus, *S. dysenteriae* is associated with the most severe symptoms of illness and generally occurs in the least developed countries. This pathogen is the only etiological agent of epidemic dysentery; between 5% and 15% of these cases are fatal, with children under the age of 5 having the highest rate of fatality. *S. sonnei* causes the least severe symptoms and is found in the more industrialized countries. *S. flexneri* usually is observed in developing countries and may cause mild to severe infections. *S. boydii* also causes mild to severe illness but rarely is isolated in developed countries and is found most often in the Indian subcontinent.

Table 46–2 Characteristics of *Shigella* species

Organisms	*Serotype*	*Geographic Distribution*	*Distinguishing Characteristics*
S. dysenteriae	Group A	Indian subcontinent, Africa, Asia	Produce Shiga toxins, cause most severe cases of dysentery, high mortality rate if untreated
S. flexneri	Group B	Most common isolate in developing countries	Less severe symptoms of dysentery
S. boydii	Group C	Rarely isolated in developed countries	Biochemically identical to *S. flexneri*, distinguished by serology
S. sonnei	Group D	Most common isolate in developed countries	Produces mildest form of shigellosis, single serotype

46.3 CHARACTERISTICS OF THE DISEASE

In general, shigellosis is a self-limiting disease, lasting 5 to 6 days if untreated; however, in young malnourished children, the elderly, and the immunocompromised (eg, acquired immunodeficiency syndrome patients),[98,118] the disease may be fatal. It is estimated that shigellosis is responsible for the death of 500,000 children worldwide each year.[8]

Clinical symptoms of shigellosis range from watery diarrhea to severe dysentery. Usually the individual has abdominal pain, which is accompanied by the appearance of mucus, blood, and polymorphonuclear leukocytes in the stool within 2 days after the onset of diarrhea.[20] Fever develops in approximately 50% of infected individuals. The illness can progress to a severe ulcerative colitis with hypotensive shock and death.[75] The incubation period following ingestion of *Shigella* is typically 1 to 4 days, depending on the strain involved and the number of organisms ingested.

The dysentery phase of illness, characterized as the passage of stools containing blood and mucus, generally is preceded by watery diarrhea 8 to 12 hours beforehand. Watery diarrhea is most likely due to jejunal secretion that is not reabsorbed by the colon because of bacterial damage to the colonic mucosa.[51,84] The dysentery stage of the disease is associated with more extensive bacterial invasion and destruction of colonic epithelial cells. As the disease progresses, areas of invasion in the colon are expanded with the spread of shigellae to adjacent cells. Areas of necrosis result in the release of dead epithelial cells and blood into the colon.[118] Organisms rarely infiltrate beyond the lamina propria, and septicemia or bacteremia seldom occur.[75] Invasion of the colonic epithelial cells appears to be necessary for illness to occur because genetically modified strains that are incapable of invasion do not cause dysentery in humans or in animal models.[28,59]

Other clinical manifestations can occur from infections due to certain *Shigella* species. Reactive arthritis or Reiter's syndrome may follow infections with *S. flexneri*.[8] Reiter's syndrome can include three clinical symptoms: urethritis, conjunctivitis, and arthritis. One possible explanation for these sequelae of shigellosis is that antibodies produced in response to antigens of *S. flexneri* cross-react with the human leukocyte antigen HLA-B27.[103] In one study, a small plasmid was reported to encode antigens structurally similar to HLA-B27.[107] Hemolytic-uremic syndrome (HUS) is caused by *S. dysenteriae* type 1 and other pathogens, notably enterohemorrhagic *E. coli* O157:H7. HUS is a common cause of renal failure in children. Although *Shigella* may be responsible for 5% to 15% of all cases of diarrhea worldwide, shigellae are associated with 30% to 50% of cases of dysentery. Other symptoms include thrombocytopenia and microangiopathic hemolytic anemia (see Chapter 43 for more detail).[48]

46.4 MECHANISM OF PATHOGENICITY

The mechanism for pathogenesis of *Shigella* infections is complex and is mediated by genetic factors similar to those of other bacterial pathogens. Much of the genetic information comprising the necessary components of *Shigella* pathogenesis is known but the function of each gene has not been completely elucidated. It is not the intent of this chapter to review in detail the complex genetics of *Shigella* virulence factors; this may be found elsewhere.[34,79]

Shigella spp. possess several key properties that are responsible for their virulence. The ingested microbes must survive the acidic environment of the stomach, which is the first significant host defensive barrier encountered by the bacteria. To produce clinical symptoms, shigellae must attach to and invade the epithelial cells of the colon, multiply intracellularly, and disseminate intercellularly through adjacent colonic epithelial cells. Although *Shigella* and most *Salmonella* spp. are enteroinvasive pathogens and share similar mechanisms of invading cultured cells, a notable difference is that internalized *Shigella* lyse endosomes and are found in the cellular cytoplasm.[89] In contrast, *Salmonella* do not lyse endosomes but multiply within them.[110]

Shigella species have a preference for M cells of the colon; these are specialized epithelial cells associated with mucosal lymphoid tissue.[80,126] After adherence to and uptake into colonic M cells, shigellae are engulfed by phagosomes and approximately 1.5 h later lyse the M cell vacuoles. The pathogen multiplies intracellularly and spreads intercellularly from the basolateral side into the submucosa of the colon. Further events are: (1) the interaction with host immune effector cells, (2) apoptotic lysis of macrophages, and (3) cytokine release and infiltration of polymorphonuclear leukocytes.[97,127]

46.4.1 Assays for Virulence

Monkeys have played a pivotal role in determining the pathogenesis of *Shigella*; however, these animals are expensive to maintain. Other animals can be used to measure certain phenotypical virulence properties of *Shigella* even though shigellae are not normal pathogens for these animals. Starved, opiated guinea pigs, fed orally with *Shigella*, have been used to show the invasive nature of this pathogen.[54] Another *in vivo* assay uses the ligated ileal loop of the rabbit to measure fluid secretion and tissue invasion.[54] The Séreny test[92] is probably the most widely used assay employed to demonstrate invasion and spread. The assay is performed by inoculating *Shigella* directly on the corneal surface of either a rabbit, guinea pig, or mouse. The development of keratoconjunctivitis indicates the ability of *Shigella* to invade the corneal epithelial cells, multiply, and spread to adjacent cells; these events mimic the invasion of colonic epithelial cells. Recently, adult rabbits were shown to display symptoms of clinical dysentery and characteristic histological changes associated with shigellosis after *S. flexneri* 2a was inoculated directly into the proximal colon.[82]

In vitro assays using mammalian cell lines, such as cultured HeLa or Henle cells, have facilitated research efforts to link biologic function to specific genetic loci of shigellae. Shigellae are able to invade and multiply within the cultured cells.[35,36,54] One version of these assays was developed to measure the ability of virulent *Shigella* to produce discrete plaques within the cultured monolayer.[76] This process parallels the invasion and multiplication stages that occur in colonic epithelial cells leading to localized infected cells and necrosis. The differential binding of the planar dye Congo Red by virulent strains of *Shigella* has been used to differentiate plasmid bearing and plasmidless cells. The genes necessary for Congo Red binding are located on the large virulence plasmid, and avirulent cells lacking this plasmid appear as white colonies on agar medium supplemented with Congo Red.[65]

46.4.2 Chromosomal Genes

The role of the *xyl-rha* region of the chromosome in intracellular multiplication of *Shigella* species was established by Falkow and colleagues using conjugational transfer of genes from *S. flexneri* 2A to *E. coli* K-12.[22] The *iucABCD* locus, which encodes aerobactin, an iron scavenger, and the *iutA* gene, whose gene product is an outer membrane receptor for this siderophore, are within this region. Aerobactin-negative mutants are invasive but exhibit a delayed reaction in the Séreny test. Most *Shigella* spp. except *S. dysenteriae* type I carry these biosynthetic and transport genes although some of these genes apparently are not expressed in *S. sonnei*.[57]

The exact role of LPS in *Shigella* pathogenesis has not been fully determined but it is thought to be a necessary component in intercellular spread to adjacent cells.[31,86] Strains that lack the O-antigen, yet still have either complete or incomplete LPS cores, are able to invade tissue culture cells but are negative in plaque assays and in the Séreny test. An important distinction between *Shigella* spp. is the location of the genes for LPS synthesis. In *S. flexneri*, the O-antigen biosynthetic genes necessary for LPS synthesis are encoded entirely on the chromosome and are located near the *his* locus. In *S. sonnei* and *S. dysenteriae*, however, synthesis of the O-antigen is plasmid-dependent. In *S. sonnei*, the 180-kbp virulence plasmid encodes a specific O-side chain of the LPS whereas in *S. dysenteriae* a 9-kbp plasmid and the large virulence plasmid are necessary for the complete synthesis of the O-side chain.

Temperature plays a critical role in controlling the expression of the virulence genes of *Shigella* spp. A chromosomal gene partly responsible for the temperature regulation of virulence gene expression is *virR* (*hns*).[66] At 30° C, *Shigella* spp. and *EIEC* are not pathogenic; on shifting the growth temperature to 37° C, these enteric organisms become virulent. Inactivation of *virR* by transposon mutagenesis yields constitutive expression of the invasive phenotype at both 30° C and 37° C.

The Shiga toxin genes *stxA* and *stxB* are near 28 minutes on the chromosomal map of *S. dysenteriae* type 1;[91] colony hybridization results indicate that other shigellae, including the other 12 serotypes of *S. dysenteriae*, do not have the structural genes for the Shiga toxin.[73] One plausible explanation why *S. dysenteriae* type 1 produces more severe clinical symptoms than other shigellae may be attributed to the action of the Shiga toxin. However, the role of the Shiga toxin in *Shigella* pathogenesis is not clear because mutants of *S. dysenteriae* type 1 lacking this toxin elicit clinical manifestations of the disease including dysentery.[25]

Shiga toxin (Stx) is a heat-labile exotoxin. The site of action of this toxin is on the vascular system of the central nervous system but it does not directly affect neurons. The toxin does inhibit protein synthesis in the host target cell. Shiga and Shiga-like toxins of *E. coli* have common properties:

1. they share sequence homology (the Shiga toxin and Stx1 from *E. coli* O157:H7 are nearly identical)
2. they have similar modes of action
3. they bind to eukaryotic cell membrane receptors with a specific galactose moiety
4. they possess the same stoichiometry of one A to five B subunits.

Shigella species also synthesize two enterotoxins—ShET1 and ShET2. The genes for *Shigella enterotoxin* 1 (*set1A*, *set1B*) are a chromosomally encoded operon and are iron-regulated. Enterotoxin ShET1 is present predominantly in *S. flexneri* 2a strains, is rarely found in other *Shigella* isolates, and is lacking in *EIEC* strains.[74] The other enterotoxin, ShET2, is plasmid-encoded and is present in *EIEC* and more than 80% of *Shigella* serotypes tested.[71] These toxins may be involved in the production of watery diarrhea in the infected host.

The virulence genes of *Shigella* are summarized in Table 46–3.

Table 46–3 *Shigella* Virulence Genes

Locus	*Function*
Chromosomal encoded	
stx	Shiga toxin
set1	Enterotoxin
virR/hns	Repressor
rfa, rfb	O-antigen biosynthesis
iuc	Aerobactin, iron acquisition
sodB	Superoxide dismutase
Plasmid encoded	
ipa	Invasion, host-pathogen interaction
mxi/spa	Secretory pathway
ipg	Chaperone
ics	Intracellular spread, actin polymerization
virF	Transcriptional regulator
virB	Transcriptional regulator
set2	Enterotoxin

46.4.3 Plasmidborne Genes

The large virulence plasmid (180 to 220 kbp) is harbored in all four pathogenic *Shigella* spp. and in *EIEC*. These plasmids share extensive DNA homology and functionally are interchangeable. Most of the plasmidborne virulence genes are in two operons oriented in opposite directions (Figure 46–1). Strains lacking this plasmid, or isolates that have deletions in the 31 kbp virulence region, are avirulent. These organisms cannot invade cultured cells or bind Congo Red in an agar medium. In *S. sonnei*, loss of the virulence plasmid not only makes the organism avirulent but also causes a change in colony morphology from Form I to Form II (smooth to rough colonies) due to loss of the LPS.[53]

The immunodominant antigens present in the outer membrane of pathogenic shigellae are the products of the four *ipa* (*invasion plasmid antigen*) genes, *ipaBCDA*. The Ipa proteins are secreted and are essential for invading colonic epithelial cells.[67,119] Extracellularly, IpaB, IpaC, and IpaA form complexes;[68] in the bacterial cytoplasm, IpaB and IpaC appear to react with the *ipgC* (*invasion plasmid gene*) gene product. The proposed role of IpaB is as a contact hemolysin, responsible for lysing the phagocytic vacuoles of the host cell[37] and inducing apoptosis in macrophages.[127] IpaD is thought to be involved in regulating Ipa protein secretion on contact with epithelial cells.[67] The *ipgC* gene, located immediately upstream of *ipaB*, encodes a protein that acts as a chaperone for IpaB and IpaC. In *Salmonella typhimurium*, homologs of IpaB and IpaC have been identified and are necessary for this pathogen to invade epithelial cells in culture.[46]

Products of 20 genes from the *mxi* (*membrane expression of invasion plasmid antigens*) and *spa* (*surface presentation of Ipa antigens*) operons are necessary for shigellae to secrete the Ipa proteins into the extracellular environment. The *mxi* and *spa* gene products share sequence homologies with the secretory proteins necessary to export the *Yersinia* outer proteins (Yops) to the cell surface[108,120] and genes responsible for flagellar synthesis in *E. coli*, *Salmo-*

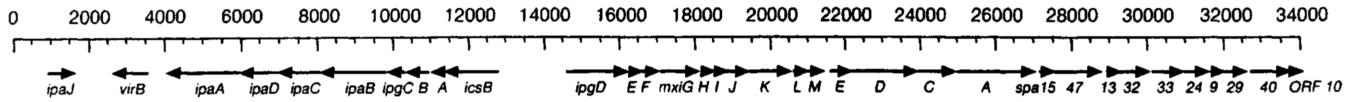

Figure 46–1 Genetic Map of *Shigella* Plasmid Virulence Genes. The map shows the location of genes necessary for *Shigella* pathogenesis on a 31 kbp region on the virulence (180 to 220 kbp) plasmid. The *ipaBCD* (*invasion plasmid antigen*) genes are the immunodominant proteins secreted by *Shigella* spp. The *mxi* (*membrane expression of invasion plasmid antigens*) and the *spa* (*surface presentation of antigens*) genes are part of the protein export system, essential for the presentation of the Ipa proteins on the cell surface. The *virB* gene is a positive transcriptional regulator. Two genes outside this region on the plasmid, *virG* or *icsA* (*intracellular spread*) and *virF*, are not shown.

nella typhimurium, Bacillus subtilis, and *Caulobacter crescentus.*[90,115]

The invasins of Gram-negative bacteria, eg, the ipa proteins of *Shigella* spp., are proteins that do not have any apparent signal peptide sequences and are dependent on a *sec*-independent Type III secretory route to be translocated to the bacterial cell surface or extracellular milieu. The genes of the secretory pathways of *Shigella* and *Salmonella* have similarities in gene order and nucleotide and protein sequence, indicating a possible evolutionary relatedness.[33,39,64] This Type III protein-secretory system also has been found in several distantly related plant pathogens such as *Xanthomonas campestris* and *Pseudomonas solanacerarum.*[23,114]

Three other virulence loci are located outside the 31 kbp region of the large virulence plasmid. The *icsA* (*intercellular spread*) or *virG* gene encodes a 120 kilodalton (kDa) outer membrane protein with adenosine triphosphatase (ATPase) activity. This protein catalyzes the polymerization of host cell F-actin monomers at one pole of the bacterium, producing an actin tail at that end. This activity propels the organism through the cytoplasm of the host cell into adjacent cells. The localization of IcsA on the outer membrane of *Shigella* appears to be dependent on intact LPS;[86] however, when the *icsA* gene alone was transferred to *E. coli*, the formation of actin tails still was observed. This led to the conclusion that the *icsA* gene may be solely responsible for intercellular movement.[31] Recently, another gene, *virA*, located upstream of *icsA*, was identified as a factor affecting invasion and intercellular spread.[113] The *virF* gene product is a positive regulatory protein and its role in *Shigella* pathogenesis is discussed below.

46.4.4 Regulation

The coordinate regulation of the virulence genes of this pathogen involves a complex cascade of molecular and physiological events. The molecular mechanisms by which *Shigella* spp. respond to environmental conditions, such as temperature and pH, have not been fully determined. *Shigella* spp. use positive and negative transcription factors to control the expression of unlinked genetic loci.

Temperature plays a critical role in regulating the expression of virulence factors of shigellae and *EIEC*. At 30° C, *Shigella* and *EIEC* are noninvasive phenotypically, but after growth at 37° C, these cells become fully pathogenic. Temperature regulation is mediated by *virR*, encoding a histone-like repressor protein (H-NS). H-NS acts by repressing the expression of the plasmidborne *virB* gene. The VirB protein, a transcriptional activator, positively regulates the expression of the *ipa*, *mxi*, and *spa* genes. The transcription of the *virB* gene is stimulated by the *virF* gene product, which binds to a region upstream of *virB*. The *virF* gene product also regulates positively the expression of the *icsA* gene at the transcriptional level. *VirF* expression apparently is not dependent on the growth temperature, although the function or conformation of the VirF protein may be affected by temperature. In a recent report, the expression of *virF* was shown to be dependent on the pH of the extracellular environment through the involvement of the *cpxA* gene.[70]

46.5 INCIDENCE OF THE ORGANISM IN THE ENVIRONMENT AND IN FOODS

S. dysenteriae was the major cause of bacillary dysentery at the beginning of the 20th century. Episodes of shigellosis caused by this pathogen decreased dramatically after World War I. *S. flexneri* then became the leading cause of shigellosis worldwide and has remained so in developing countries. After World War II, *S. sonnei* was the most frequent isolate recovered from patients in developed countries. *S. sonnei* remains the leading *Shigella* spp. isolated from humans with shigellosis in the US. In 1969, *S. dysenteriae* reappeared in Guatemala; this pathogen has become endemic in Africa and Asia. Considerable numbers of shigellosis outbreaks caused by *S. boydii* are found in India (see Table 46–2).

The primary route of transmission of *Shigella* spp. is the fecal-oral route. In developing countries, the spread of these pathogens is sometimes rapid due to contamination of local water supplies. In developed countries where proper sanitation exists, the dissemination of shigellae remains a public concern. Poor personal hygiene of food handlers, whether in large institutions or in small hometown cookouts, contributes significantly to several outbreaks each year. Outbreaks in day-care centers and nursing homes have become very common. In the former, toddlers have not developed good toilet training habits and easily can spread the pathogen to other children or adult supervisors.

46.6 EPIDEMIOLOGY

46.6.1 Incidence of Outbreaks and Sporadic Cases

There are three major routes for contracting shigellosis: person-to-person contact, ingestion of contaminated water, and ingestion of contaminated food. Person-to-person spread of the organism is the most common route for the spread of shigellae. This pathogen can be disseminated from infected individuals by flies, fingers, food, or feces.[32] Houseflies are considered passive vectors and can be responsible for epidemic dysentery. Shigellae are capable of passing through the gut of flies and can be transported directly by flies to foods from contaminated feces. Shigellosis outbreaks are seasonal; higher incidence occurs during warmer periods of the year. Because the infectious dose of shigellae is very low, the likelihood of developing clinical shigellosis and rapid dissemination of this pathogen within a given population is high.

A major cause of small- or large-scale foodborne outbreaks is poor personal hygiene of a food handler. Cool, moist foods that require extensive handling after the ingredients are cooked, such as egg or potato salads, are good vectors for spreading shigellae from infected food handlers to unsuspecting consumers. The second-leading cause of foodborne outbreaks of shigellosis is the improper storage of contaminated foods;[102] contact with contaminated equipment, inadequate cooking, and unsafe food sources are other contributing factors.

A decrease in US foodborne outbreaks of shigellosis has been reported to the Centers for Disease Control and Prevention from 1983 to 1992. In 1983–1988, there were 110 foodborne outbreaks of shigellosis with 17,990 cases; whereas in 1992, only 1 outbreak and 4 cases were reported. The World Health Organization (WHO) tracks foodborne outbreaks caused by a number of bacteria, including *Shigella* spp. in Europe and several Asian countries.[124] In the years 1985–1989, Hungary reported 7 shigellosis outbreaks, Romania had 2 outbreaks with 73 cases, the former Yugoslavia counted 6 outbreaks, and Spain 13 outbreaks. In Israel, 25% of all foodborne outbreaks were caused by shigellae. Twenty-seven outbreaks with 11,098 cases were reported during this period.

Members of the genus *Shigella* have a limited host range, which is restricted mainly to humans and higher primates, including gorillas, gibbons, and macaques. Frequent outbreaks are mostly associated with zoological (conservation) parks and primate research centers with *S. flexneri* as the primary causative agent, and *S. sonnei* infrequently isolated.[5,60,106] In some instances, animal caretakers have contracted shigellosis by tending monkeys shedding shigellae.[50]

Examples of Foodborne Shigellosis Outbreaks

The following outbreaks of shigellosis indicate that the spread of *Shigella* from contaminated foods to unsuspecting individuals can occur under many different circumstances.

1994: Shigellosis in Europe. An outbreak of shigellosis occurred in Norway, the United Kingdom, and Sweden. The probable cause was the ingestion of fecal-contaminated iceberg lettuce imported from Spain. Although *S. sonnei* was not recovered from this food, a well-documented case-control study implicated imported lettuce. The coliform count found in lettuce samples was up to 10^8 cfu g^{-1} indicating fecal contamination, most likely due to irrigation practices using poorly treated sewage water.[47]

1992-1993: Operation Restore Hope in Somalia. Diarrheal diseases were experienced among US troops stationed in Somalia participating in Operation Restore Hope. The leading cause of diarrhea among personnel was *Shigella* spp. (33%), followed by enterotoxigenic *E. coli* (16%) and *Giardia lamblia* (4%). *S. sonnei* was linked to 10 cases that involved a common eating facility.[95]

1992: Shigellosis outbreak at a central commissary in Michigan. A review of the premises at a commissary in Michigan revealed that several food handlers were ill before an outbreak of shigellosis that involved 43 individuals. *S. flexneri* was the causative agent, and tossed salads were the suspected food vehicle.[18] This scenario typifies several aspects of infections due to *Shigella*: the relative ease with which *Shigella* can spread through a population, uncooked vegetables as a common vehicle for dissemination of shigellae in foods, and the contribution of food handlers with poor personal hygiene to the transmission of the pathogen. The need for food establishments to monitor food handlers is well recognized as a critical factor to be addressed.

1991: Shigellosis caused by moose soup. At a community festivity in Galena, Alaska, 17 of 19 case patients with gastroenteritis ate homemade moose soup. *S. sonnei* was isolated from 10 of these patients. The soup was prepared in five different homes; in one household, one person complained of having a gastrointestinal problem either before or during the preparation of the soup. Because no other food was implicated in the outbreak, this would indicate that the contaminated moose soup was prepared from one of these households.[29]

1988: Large shigellosis outbreak at a music festival. At an outdoor music festival, approximately 3,175 people reported a diarrheal disease caused by *S. sonnei*. A total of 6,403 people attended this gathering. A week before the festival commenced, gastroenteritis was reported among the 563 staff members. It appears that the transmission of the infective agent to attendees was initiated by ill staff members. Contributing factors to this large outbreak were lack of proper sanitary conditions (lack of adequate soap and handwashing facilities) and the large number of food handlers (2,000 people).[58]

1987: Large shigellosis outbreak at a mass gathering. More than 12,700 people attended a mass gathering where more than 50% of the attendees experienced a diarrheal episode. The mode of transmission was most likely food, water, and person-to-person contact. Sanitary conditions were poor with kitchen facilities located near open latrines. Exacerbating the problem was the fact that food handlers with gastroenteritis were not excluded from food preparation. An antibiotic-resistant (ampicillin, tetracycline, and trimethoprim-sulfamethoxazole) strain of *S. sonnei* was identified as the causative agent.[121]

Examples of Waterborne Shigellosis Outbreaks

1991: Shigellosis in Thailand. Shigella flexneri 2a was responsible for an outbreak of shigellosis in Thailand. This waterborne outbreak was most likely due to a piped public water supply with an interruption in chlorine treatment and subsequent contamination with fecal matter.[109]

1991: Lake contaminated with Shigella. Over a 24-day period at a lakeside park near Portland, Oregon, 59 people had bloody diarrhea caused by either *S. sonnei* or *E. coli* O157:H7; the latter pathogen also was responsible for several people developing HUS. Consumption of food or beverages was not implicated in the spread of the disease. The fecal coliform count in the lake was substantial. *E. coli* O157:H7 isolated from lake water samples were identical to patient isolates as determined by pulse-field gel electrophoresis.[49]

1990-1991: Outbreak of shigellosis on the Island of Crete. Nearly one-tenth of the 1,479 inhabitants of one village on the Island of Crete had diarrheal disease, with 30% of the children under the age of 12 years being symptomatic. *S. sonnei* was isolated from stool cultures, and the mode of transmission was most likely due to contaminated spring water.[85]

46.7 FACTORS AFFECTING SURVIVAL AND GROWTH IN FOODS

Shigella, unlike other foodborne pathogens such as *Salmonella* and *Listeria*, are not indigenous to one particular food. The addition of *Shigella* to foods is usually the consequence of a food handler with poor personal hygiene; however, raw vegetables and fruits can be tainted with contaminated water either used for irrigation or washing. Examples of foods that have been implicated in outbreaks are potato salad, tossed salads, spaghetti, egg salads, white cheese, tuna salad, stewed apples, chicken, shrimp, clams, and milk.[102]

Studies on the survival and growth of shigellae in foods have focused mostly on *S. sonnei* and *S. flexneri*, with some work reported with *S. dysenteriae*. Much of this information has been reviewed by the International Commission on Microbiological Specification for Foods,[42] and some of those data are presented below.

The ability of shigellae to survive in foods can depend on a number of factors. The temperature at which the food is held, the pH, and presence of inorganic acids in foods have a critical part in the survival of this pathogen.

Shigellae can survive over a range of temperatures. *S. sonnei* can grow in nutrient broth at temperatures as low as 6° C (*S. flexneri* as low as 7° C). In foods, *S. flexneri* and *S. sonnei* can survive when stored at –20° C, 4° C, and room temperature when exposed to 80° C for a few seconds. The length of survival is quite different for each temperature, with shigellae surviving for the longest period at room temperature.

The chances of recovering shigellae from foods seem to be better in foods with neutral pH—eg, margarine and butter—because these bacteria survive for long periods (greater than 120 days) even at temperatures at –20° C and 4° C. In acidic foods, such as citric juices and carbonated soft drinks, *S. flexneri* and *S. sonnei* can survive from 4 h to 10 days. *S. dysenteriae* was tested for survival in orange and grape juices at –4° C and 20° C, respectively, and was found to survive from 2 to 28 hours in grape juices and up to 170 hours in orange juice. *S. sonnei* did grow in media with pH values between 4.8 and 9.3, and *S. flexneri* had nearly the same pH range, from 5 to 9.2.

In foods that have been implicated commonly in shigellosis outbreaks, such as cheese, potato salad, and mayonnaise, *S. sonnei* and *S. flexneri* can survive at 4° C for 21 days. In Greek cheese, *S. sonnei* can survive from 24 to 92 days depending on the storage temperature; at 4° C, this bacterium survived the longest.

The presence of organic compounds, such as formic or acetic acid and salts, eg, NaCl, has a pronounced affect on survival of shigellae in different conditions. No growth was observed in media containing 3.8% to 5.2% NaCl with a pH of 4.8 to 5.0. In media with nitrite concentrations of 300 to 700 ppm at a pH of 5.5, these bacteria did not grow. Overall, *S. flexneri* is more sensitive to inhibitors than *S. sonnei*. *S. flexneri* was less able to grow in the presence of nitrites, salt, and pH at lower temperatures.

In one report,[83] *S. flexneri* survival was tested in several vegetables, either sterile or nonsterile. In either case, the level (10^5 to 10^6 cfu g^{-1}) remained constant for several days. There have been a few outbreaks of shigellosis caused by the ingestion of contaminated vegetables,[16] indicating that *Shigella* can survive in sufficient numbers to produce clinical symptoms. The ability of *Shigella* spp. to survive acid conditions in foods has been sparsely investigated. Two reports[7,104] demonstrated that the number of *Shigella* inoculated into meats was reduced after treatment with formic or lactic acid.

Acid resistance by shigellae appears to be mediated by the *rpoS* (*katF*) gene, a locus encoding for an RNA polymerase sigma factor.[99] A model of gastric acid resistance suggests that this sigma factor controls the expression of genes under duress, such as poor growth conditions (eg, low temperature, depleted nutrient supply); thus, the genetic factors for acid resistance are expressed along with a battery of other survival characteristics.

46.8 PRINCIPLES OF DETECTION AND ISOLATION FROM FOODS

46.8.1 General Comments

Shigella spp. in foods are a potentially serious problem to the public health. Rapid detection of contaminated foods is critical because of the low infectious dose and the potential for rapid dissemination of these pathogens. Contamination of foods usually occurs through a food handler with poor personal hygiene.[102]

Analytical epidemiological studies are often the only way to determine the vehicle of transmission in outbreaks of disease when perishable commodities are involved. Furthermore, most outbreaks of shigellosis are recognized days or weeks after exposure or onset of the disease. Analysis of suspected foods may be further compromised by improper storage conditions (abused by temperature or time) or loss of the food sample (disposed or eaten).

In foodborne outbreaks caused by *Shigella* spp., the initial recognition of shigellosis usually is based on the results from examining patient samples in the clinical laboratory. Detection of *Shigella* in this milieu is enhanced by two significant factors: humans are efficient incubators for this pathogen and can shed considerable numbers of *Shigella* in stools, and there is usually only one type of clinical sample to be analyzed, ie, stool specimens. Therefore, clinical identification schemes based on standard bacteriological means are fairly straightforward. This luxury is not present for the food analyst.

Foods vary greatly in composition (pH, fat, and oil content) and the indigenous microbial flora. The food analyst must consider these factors to implement the correct scheme for detecting shigellae in foods. An accurate diagnosis of the presence of shigellae in foods usually is inconclusive because of the low numbers or injured state of the *Shigella* spp. present and further complicated by the composition and consistency of the food sample.

The variability of the indigenous microbial load of each food poses a significant problem for detecting *Shigella* in foods whether by bacteriological methods or by DNA-based assay systems. For instance, some foods, such as alfalfa sprouts, have a microbial flora of 10^7 to 10^8 cfu g^{-1},[55] and the indigenous population can easily outcompete and therefore outgrow any *Shigella* spp. present. The problem of having a high bacterial population and a low number of *Shigella* can affect the successful recovery of *Shigella* spp. from foods. Because of its low infectious dose, *Shigella*, even in undetected levels in foods, still poses a significant public health risk.

Detecting or recovering shigellae in foods is a daunting task with a low probability of success. Smith[102] has commented on the resuscitation of injured (or abused) shigellae from foods and the low chance of recovery. Implementing DNA-based techniques may increase the chance of detecting shigellae in foods but, to date, these methods have not been tested rigorously in foods.

The use of probes for the detection of shigellae may be limited. In many foods, the microbial flora are present in higher numbers than the shigellae; therefore the *Shigella* spp. present would not be represented on the agar plates after diluting the samples to yield an appropriate number of cfus per plate. The polymerase chain reaction (PCR) may be a preferred detection method rather than using hybridization protocols with gene probes. The number of target cells for PCR is between 10^2 and 10^3 whereas for probes 10^3 to 10^5 cfu g^{-1} is needed. The major hindrance for PCR detection is the preparation of template DNA; inhibitors of the PCR from the food sample must be removed and enough template must be available to be amplified in sufficient quantity.

46.8.2 Standard Bacteriological Methods

Conventional bacteriological methods for the recovery and identification of *Shigella* spp. from foods can take 7 to 10 days. Suspected food samples are grown initially in enrichment broth followed by biochemical tests for preliminary identification and subsequent serological assays for confirmation. The *Bacteriological Analytical Manual*[2] details how some government agencies and food companies analyze food samples. An enrichment step includes adding a portion of the food to Shigella broth supplemented with 0.5 to 3 $\mu g\ ml^{-1}$ of novobiocin. This antibiotic appears to inhibit many Gram-negative bacteria but does not affect the growth of *Shigella*. The broth culture is grown anaerobically at 42° C to 44° C for 20 hours and then streaked onto MacConkey agar plates. After growth at 35° C for 20 hours, suspected lactose-negative colonies are examined by Gram-stain and tested for specific biochemical reactions (eg, H_2S, urease, lysine decarboxylase, citrate) and subsequently tested by group-specific antiserum. Shigellae are thought to be nonmotile, and motility agar is used as a diagnostic indicator; however, one report suggests that *Shigella* spp. may be minimally motile.[30] Therefore, this test may be unsatisfactory for identifying all *Shigella*.

Different bacteriological methods also are available for identification of *Shigella* spp. Food samples also can be grown in enrichment broth cultures of Gram-negative or selenite cystine broth.[4] Overnight cultures can be plated onto several different selective media including MacConkey, *Shigella-Salmonella*, xylose-lysine-deoxycholate, eosin-methylene blue, or deoxycholate citrate agar plates. Another scheme uses growth on either triple-sugar iron or Kligler iron agar slants, and suspected cultures are again identified using biochemical and serological criteria.

The enumeration and identification of foodborne microbes using standard microbiological regimens is labor intensive, time consuming, and lacks the specificity and sensitivity needed to detect these pathogens in foods.[2] With the techniques of immunobiology and molecular genetics, alternative methods such as DNA probes, PCR, and monoclonal antibodies can be applied to the development of detection assays for foodborne pathogens.

46.8.3 Genetic and Immunological Methods

46.8.3.1 DNA-Based Assays

The first gene probes developed to detect *Shigella* targeted these pathogens in clinical samples.[10,100,111,123] DNA fragments were isolated from the large virulence plasmid resident in pathogenic strains of *Shigella* and used in a colony hybridization format to discriminate between virulent (retaining the virulence plasmid) and avirulent (cured of the virulence plasmid) strains. Table 46–4 summarizes the DNA probes used to target specific regions of the large virulence plasmid.

In one study, a specific oligonucleotide was synthesized based on the sequence of the repeated *ipaH* genes and used as a probe to detect *Shigella* spp. and *EIEC* isolated from clinical samples.[116,117] Copies of the *ipaH* genes are located on the *Shigella* chromosome and on the large virulence plasmid, which makes this oligonucleotide a useful genus identification probe. A comparison was made using an alkaline phosphatase-conjugated oligonucleotide probe for the *ipaH* gene and the Séreny test in identifying virulent strains of *Shigella* spp. These comparisons were made using cultures isolated from clinical

Table 46–4 DNA Probes and PCR Primer Sets for Detecting *Shigella*

DNA Probes

Probe	*Type*	*Application*	*Reference Number*
17 kbp	Plasmid DNA	Clinical	21,111
2.5 kbp	Plasmid DNA	Clinical	93,100,123
ipaBCD	Plasmid DNA	Clinical	116
ipaC	Oligonucleotide	Clinical	117
paH	Oligonucleotide	Clinical	117
ial	Oligonucleotide	Clinical	63
ial	Oligonucleotide	Food	45

PCR Primer Sets

Target	*Application*	*Reference Number*
ial	Clinical	26
ial	Food	56
ipa	Clinical	117
virF	Clinical	125
uidA	Clinical	6,14
phoE	Clinical	105

samples;[117] however, avirulent strains (plasmid-less) of *Shigella* and *EIEC* also can be detected with this probe.

In contrast, for food analysis, a synthetic 18-base long oligonucleotide, based on the nucleotide sequence of a virulence gene found in a 2.5 kbp HindIII fragment from the invasion plasmid[90,100,111] was used as a probe. Although several genes have been identified in this 2.5 kbp fragment, this oligonucleotide probe targeted the *ial* (*invasion associated locus*)[101] also known as ORF8.[90] The oligonucleotide probe was tested for specificity, with *Shigella* and *EIEC*, and sensitivity, in several foods seeded with *S. flexneri*.[45] The efficacy of this probe was dependent on the seeded food. *S. flexneri* seeded (10^2 cfu g^{-1}) in alfalfa sprouts could not be detected by colony hybridization; the microbial background (1.4×10^8 cfu g^{-1}) of this particular food apparently outgrew the seeded *Shigella*.

DNA probes for the detection of pathogenic *Shigella* and *EIEC* in foods using the colony hybridization blot format suffer other disadvantages. In cases where food samples are time or temperature abused, an enrichment step in broth cultures may not resuscitate the requisite number of cells to detect by hybridization. The low infectious dose of *Shigella* means that contaminated foods may fall below this level of detection, ie, give a false negative result. Also, analyzing foods with DNA probes, as with other bacterial cultural methods, is time consuming and laborious. The application of PCR has overcome some of the disadvantages of DNA probe and culture methods and has reshaped the direction for developing methods for the detection of *Shigella* spp. and *EIEC*, not only in the clinical laboratories but also in food analysis.

Several PCR primer sets directed against known virulence markers, such as the *ial* locus (ORF8),[26,56,62] the *ipaH* gene,[94,117] and the *virF* locus[125] and genes not associated with pathogenesis, eg, *uidA*, *phoE*, have been tested in a PCR assay.[6,14] Most of these PCR assays were tested with clinical or water samples. Table 46–4 summarizes PCR primers used to detect *Shigella* in either clinical or food samples.

Another method combines PCR with immunomagnetic separation to detect *S. dysenteriae* type 1 and *S. flexneri* from feces.[43] The monoclonal antibodies used in this assay target a common epitope of the O-polysaccharide of both pathogens and were used to coat magnetic beads. Bacteria that bind to the beads directly from stool samples are used as templates in a PCR assay specific for these *Shigella* species using *ial*-specific PCR primers. The reported sensitivity of a combined immunomagnetic separation, PCR, and dot blot hybridization was 10 to 15 shigellas g^{-1} of fecal sample.

For epidemiological analysis, molecular typing methods have been compared to determine the best technique to type *S. sonnei* isolates. Plasmid profile, restriction endonuclease digestions of plasmids, rRNA gene restriction (ribotyping), pulsed-field gel electrophoresis, and enterobacterial repetitive intergenic consensus (ERIC) sequence-based PCR analysis were used; the last showed the most promise for typing *S. sonnei*.[61]

Clinical samples are rather consistent in composition and the number of *Shigella* cells shed in positive stool specimens can be high. The physical composition of each food, eg, fats, oils, may have an adverse affect on the PCR chemistry. Therefore, unlike the clinical sample, each food matrix may pose a unique challenge in extracting a suitable number of target DNA molecules from *Shigella*.

PCR has been used to analyze food samples that were seeded with either *S. flexneri*[56,83] or *EIEC*.[1] PCR primers were selected to target the *ial* gene (ORF8). PCR products of the appropriate size, as visualized by gel electrophoresis, were amplified from seeded foods. With improved DNA isolation techniques and faster thermocyclers, it is possible to detect *Shigella* in foods in less than 8 h. PCR-based assays are rapid and sensitive and do not require selective enrichment or high cell numbers.

PCR-based assays do have their disadvantages. As in any PCR system, template DNA from live and dead cells can yield positive results. But in the case of *Shigella*, these bacteria are not indigenous to any food and it can be argued that a positive result from PCR indicates contamination with human fecal matter. A limitation of a PCR assay that targets a plasmid-encoded virulence factor for detecting *Shigella* in foods is that the loss of the virulence plasmid renders PCR (and probe) methods ineffective. With *S. sonnei*, this is a problem because these pathogens lose the virulence plasmid quite readily during isolation and storage.[65,88] PCR primers for the *ipaH* gene can overcome the problem of plasmid loss because this genetic marker is also on the chromosome. However, because plasmid-less strains are avirulent, targeting the *ipaH* gene may not satisfy regulatory concerns of governmental agencies.

The incidence of antibiotic-resistant shigellae is increasing worldwide and is a great concern to the medical community in treating outbreaks. To increase the chance of recovery and detection of *Shigella* in foods by genetic-based assays, antibiotics can be incorporated into enrichment medium or in agar plates. This effectively reduces the microbial flora of the food sample and maintains selective pressure on the *Shigella* spp. for that particular antibiotic. Because most shigellosis outbreaks are identified initially in the clinical laboratory, the choice of antibiotic to use is dependent on the antibiotic resistance profile of the isolated pathogen. This information can be most useful to the food analyst in identifying the source of the foodborne outbreak. This method has proven effective with foods, such as alfalfa sprouts and ground beef, that have high background flora [A.-A. Molina and K.A. Lampel, unpublished observation].

46.8.3.2 Latex Particle Agglutination Assays

For clinical samples, several commercial latex agglutination tests are available for the detection of *Shigella* spp. A color-coded latex kit (Wellcolex Color Shigella Test; Wellcome Diagnostic Research, Triangle Park, NJ) and the Bactigen Salmonella-Shigella Latex Agglutination Slide Test (Wampole Laboratories, Cranbury, NJ) were tested for specificity and sensitivity using enrichment broth cultures of clinical samples and direct culture identification.[17,69] The specificity of both latex agglutination methods ranged from 93.6% to 99.2%, although it should be noted that the number of samples used in the slide-test method was quite low. The sensitivity of the color-coded latex kit was 97.1%; with pure cultures, 98%.[11]

A hydrophobic latex particle agglutination assay that can differentiate virulent, plasmid-bearing *Shigella* spp. from avirulent, plasmid-less strains has been reported.[9] Although this method is rapid, its efficacy in detecting *Shigella* in food samples has not been determined.

46.8.3.3 Other Antibody-Based Assays

Enzyme-linked immunosorbent assay (ELISA). One ELISA test uses antibodies raised against an *EIEC* "virulence marker antigen" and was shown to identify clinical isolates of *Shigella* and *EIEC.*[77] This application, although it could screen a large number of clinical isolates in 24 hours, was tested using pure isolates and has not been applied to detecting *Shigella* from foods.

Immunomagnetic. An immunomagnetic assay using monoclonal antibodies directed against the O-antigens of *S. dysenteriae* type 1 and *S. flexneri* was tested using clinical samples and pure cultures.[44] The sensitivity of this assay was shown to be 10^3 cfu ml^{-1} with a specificity of 100%; the assay could detect *Shigella* in stool specimens up to 2 days after antibiotic therapy was initiated in patients.

46.9 CONTROL

Control of shigellosis is best achieved by preventing food and water from becoming contaminated with the microbes. Many food inspection systems have relied on good hygienic practices in food manufacturing and preparation facilities and used endproduct sampling to monitor food safety. This approach to food safety is being replaced worldwide by the Hazard Analysis Critical Control Point (HACCP) approach,[41] a process control system based on identification of points at which biological, chemical, and physical hazards may be introduced into a food. The HACCP approach is addressed in Chapter 58 and will not be discussed in detail here. It is, however, a much more scientifically based approach to ensuring food safety when compared to endproduct sampling. This is especially true when dealing with microbes with low infectious doses such as members of the genus *Shigella*.

The prevention of shigellosis can be enhanced by the application of better personal hygiene among food handlers and consumers. This includes handwashing and, in many countries, improvement in water treatment and better monitoring of food handlers, day-care personnel, and children. Industries employing food handlers and day-care center personnel should have testing of stool samples from infected individuals until negative results are obtained. This policy is in effect in many local jurisdictions in the US.

The use of antibiotic therapy should be dictated by the severity and stage of the disease. In some cases, such as with *S. sonnei*, the disease is self-limited and antibiotic treatment may be unnecessary. However, in more severe cases with bloody diarrhea, dispensing of antibiotic to the patient may reduce the length of the illness. Antibiotics recommended include trimethoprim/sulfamethoxazole, ampicillin, and, in situations with drug-resistant strains, nalidixic acid or 4-fluoroquinolones. A concern of many scientists is that multiple-resistant strains are evolving globally and indiscriminate use of antibiotics may contribute to the spread of antibiotic resistance genes in the bacterial world; therefore, therapy should always follow antibiotic sensitivity testing.

Resistance to antimicrobials can occur quickly, as in the case of *S. dysenteriae*, and has been observed even during a single epidemic. In developing countries, this can be a major obstacle for treatment; antibiotics available locally may no longer be effective, and more expensive alternative antibiotics, which are not on hand, may be needed. Bacterial extrachromosomal plasmids carrying genetic determinants are responsible for dissemination of resistance but the mechanisms for the rapid spread of antibiotic resistance observed with *S. dysenteriae* are not completely known.

The rising incidence of shigellosis outbreaks and the number of antibiotic-resistant *Shigella* encountered continue to be worldwide concerns. The latter problem is not restricted to *Shigella* but is present in a vast number of important human pathogens. As suggested by Høiby et al.,[38] the spread of shigellae via foods and their possible exposure to and selection by antibiotic regimens in clinical environs may add to the antibiotic resistance repertoire of these pathogens. Subsequent dissemination of these strains to other individuals will only increase the persistence of *Shigella* spp. in the world. The WHO has recognized the importance of antibiotic resistance by pathogens such as shigellae. This organization has established a monitoring program to track resistance on a national and regional basis and also to provide guidelines for a rational approach to the use of antibiotics.

No vaccine currently is available; however, there have been several reports recently published on the progress of developing a vaccine for all *Shigella* spp.[81] The WHO specifically has targeted this pathogen for a program leading to a successful vaccine and has supported research for elucidating the molecular and cellular events of *Shigella* pathogenesis.

46.10 CONCLUDING REMARKS

Diarrheal diseases are a leading cause of death of children worldwide. As one of the major diarrheal diseases, shigellosis remains a concern, occurring frequently in developed and developing countries. Improved sanitary conditions and education in better hygiene practices are important factors in reducing the number of incidences of bacillary dysentery. Also, as recognized by the WHO, research to develop a vaccine against *Shigella* spp. and to unravel the genetics of the molecular and physiological mechanisms of host-pathogen interactions can contain the spread of shigellosis. The fact that shigellae have shown a rapid increase in antibiotic resistance makes all these preventive measures increasingly important to those areas in the world where diarrheal diseases lead to high mortality.

Finally, it is incumbent on food microbiologists, the food industry, and the food regulatory agencies to ensure a safe food supply for the consumer. This applies to developing and industrialized nations. Improved methods for the rapid detection of shigellae in foods may facilitate the prevention of the spread of this pathogen.

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47 Staphylococcus aureus

Tony C. Baird-Parker

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References

47.1 INTRODUCTION

The name staphylococcus, derived from the Greek nouns *staphyle* meaning a bunch of grapes and *coccus* meaning a grain or berry, was first used in 1882 by Sir Alexander Ogston to describe the cluster-forming cocci he observed in pyogenic abscesses in man.[115] Two years later, Rosenbach reported the isolation of this organism in pure culture and introduced the name *Staphylococcus aureus* for the orange colony-forming cocci.[128] Over the next 75 years staphylococci were placed by microbial taxonomists in a variety of tribes and families, including the Coccaceae, Metacoccaceae, Streptococcaceae, Micrococceae, and the Micrococcaceae. *Staphylococcus aureus* also was given a number of other specific epithets including *Staphylococcus pyogenes* var. *aureus*, *Micrococcus pyogenes*, and *Micrococcus aureus*.[10,88] *Staphylococcus aureus* is currently the type species of the genus *Staphylococcus*.[30] The genus *Staphylococcus* is a well-defined group of microorganisms that, with the exception of morphology, shares few characteristics with the genus *Micrococcus*. This latter genus has been recently renamed *Kocuria* and is most closely related to the arthrobacters, whereas the genus *Staphylococcus* is most closely related to the genera *Planococcus*, *Enterococcus*, and *Bacillus*.[88]

Although one of the first recorded likely associations of staphylococci and foodborne illness was in 1884 and involved an outbreak of food poisoning associated with cheese,[28] it was not until 1914 that Barber showed that *S. aureus* caused illness by consuming milk from a cow with staphylococcal mastitis.[17] The involvement of a toxin later was shown by Dack and his colleagues, who, in 1930, reported that feeding sterile filtrates of a culture of *S. aureus* to human volunteers resulted in typical symptoms of staphylococcal food poisoning.[46] Since the 1950s, *S. aureus* has been shown to produce a number of antigenically distinct enterotoxins. Up to 1996, 10 were recognized.[171]

47.2 TAXONOMY AND TYPING

47.2.1 Taxonomy

Staphylococci are nonmotile, Gram-positive, and catalase-positive cocci dividing in more than one plane to form irregular three-dimensional clusters of cells. They are facultative anaerobes and use glucose by glycolysis and use of the hexose monophosphate pathway.[88] The separation of staphylococci from micrococci on the basis of their chemical and biochemical characteristics is shown in Table 47–1.

Staphylococcus aureus can be separated from other species of staphylococci by a series of biochemical and resistance characteristics. Table 47–2 shows the principal characteristics separating the main species of staphylococci. For a description of the characteristics of all 31 species recognized in 1992 see Kloos et al.[88]

Staphylococcus aureus usually ferments mannitol and, in the presence of air, uses a wide range of hexoses, pentoses, and sugar alcohols; lactic acid and acetoin are the main end products of glucose metabolism. Other enzymatic activities typical of *S. aureus*, and some closely related species, include: coagulation of animal plasmas by staphylocoagulases and the clumping factor; production of alkaline phosphatase and heat-resistant nuclease (TNase); and production of staphylokinase (fibrinolysin) and a large variety of cytotoxins and cytolysins.[6,88] Staphylococcal enterotoxins are produced mainly by *S. aureus* strains isolated from man (see Section 47.6).

The cell wall of *S. aureus* contains three main components:

1. A peptidoglycan comprising repeating units of N-acetylglucosamine beta-1,4 linked to N-acetylmuramic acid, with the carboxyl group of the muramic acid substituted by an oligopeptide with adjacent units of the peptide bridged by a pentapeptide containing solely or mainly glycine to form a three dimensional structure[131,152]
2. A ribitol teichoic acid bound via (glycerol-phosphate) N-acetylmannosaminyl-beta-1,4-N-acetylglucosamine to a muramyl-6-phosphate residue and composed of N-acetyl-D-glucosaminyl-D-ribitol phosphate in which the N-acetyl group is either 4-O-alpha or -beta linked[89]—the latter corresponds to the group-specific Polysaccharide A[114]
3. Protein A, which is covalently linked to the peptidoglycan and particularly is characterized by its ability to bind with the Fc component of the immunoglobulin of many mammalian plasmas causing autoagglutination.[107]

47.2.2 Typing

The classic method of distinguishing strains of *S. aureus* is to use their relative susceptibility to infection and lysis by different staphylophages, ie, by phage typing. While the pattern of lysis by phages has little or no epidemiological significance, the pattern of lysis to a range of phages is a useful means of distinguishing strains from a particular environment or identifying the possible source of a particular type.[162] There is an internationally agreed basic set of phages used for typing strains of *S. aureus* of human origin, which is updated regularly to reflect changes in the typability of human clinical isolates. Phages additional to the basic set may be used for particular purposes, such as the separation of strains from particular environments. Strains isolated from animals

Table 47–1 Chemical and Biochemical Characteristics Separating Staphylococci from Micrococci

Character	*Staphylococcus*	*Micrococcus*
Nucleic acid·		
mol% G+C in DNA	30–39	64–75
Cell wall		
Teichoic acid	+	–
Glycine in interpeptide bridge of peptidoglycan	+	–
Respiratory chain		
Cytochromes c and d	–	+
Major menaquinones	MK-6to8 (unsaturated)	MK-7(H_2)to9(H_2) (partially hydrogenated)

Source: Adapted from ICMSF, *Staphylococcus Aureus*, in *Microorganisms in Foods 5: Characteristics of Microbiological Pathogens*, © 1996, International Commission on Microbiological Specifications for Foods (ICMSF).

Table 47–2 Characters Separating the Main Species of Staphylococci

Character	*S. aureus*	*S. intermedius*	*S. chromogenes*	*S. hyicus*	*S. epidermidis*	*S. capitis*	*S. hominis*	*S. warneri*	*S. haemolyticus*	*S. cohnii*	*S. saprophyticus*	*S. carnosus*	*S. xylosus*
Production of:													
Coagulase*	+	+	–	+/–	–	–	–	–	–	–	–	–	–
TNase	+	+	–	+	–	–	–	–	–	–	–	–	–
Hemolysis†	+	V	–	–	–	–	–	–	–	–	–	–	–
Acetoin	+	–	–	–	+	V	V	+	+	V	+	+	V
Acid, aerobically from:													
Sucrose	+	+	+	+	+	+	+	+	+	–	+	–	+
Trehalose	+	+	+	+	–	–	+	+	+	+	+	V	+
Mannitol	+	+/–	+/–	–	–	+	–	V	V	+	+	+	+
Alkaline phosphatase	+	+	+	+	+	–	–	–	–	–	–	+	V
Novobiocin (1.6 μg ml^{-1})	S	S	S	S	S	S	S	S	S	R	R	S	R

–: >80% –ve; +: >80% +ve; +/–: weak; V: variable; S: sensitive; R: resistant
*rabbit plasma
†bovine/human blood

Source: Adapted with permission from A.C. Baird-Parker, Methods for Identifying Staphylococci and Micrococci, in *Identification Methods for Microbiologists*, 2nd ed., The Society for Applied Bacteriology Technical Series No. 14, pp. 201–210, © 1979, Academic Press, Inc.

are mainly untypable by the use of phages used for typing human strains, so other sets of phages are used for typing strains from food animals.[47,104,106] A number of workers have recognized the limitation of phage typing schemes. There are problems of nontypability (15% to 20% of isolates may be untypable) and variation in typability caused by lysogeny and the often poor typability of strains endemic in hospitals.

Antigenic typing, while used by some, has not gained wide acceptance because of antigenic complexity, variability, and the difficulty in preparing specific antisera.[11] However, the combination of antigenic and phage typing is useful in epidemiological and other studies.[43,51]

A variety of molecular probe-based methods have been proposed for typing *S. aureus*, including plasmid profile typing[132] and ribotyping.[32,53] Pulsed-field gel electrophoresis (PFGE) combined with plasmid profile typing was found to be particularly useful for typing *S. aureus* isolates from foods;[156] however, these methods are not widely used at present and it remains to be seen whether any of them will replace phage typing as a routine typing technique. They cannot, at present, compete with phage typing on either cost or throughput rate, although, ultimately, genotyping is likely to be the preferred method for typing strains of *S. aureus*.

A number of attempts have been made to group *S. aureus* strains according to their biochemical and physiological characteristics. Among the most developed of these is that proposed by Hajek and Marsalek.[73] They were able to group *S. aureus* strains from different animal species into six biotypes, using a range of characteristics that included staphylokinase, beta hemolysin, bovine plasma coagulation, and growth on crystal violet agar. This classification has been subjected to a number of modifications as a result of additional studies by other workers, and seven biotypes (A-G) are listed by Devriese.[49] Two of these biotypes (Hajek and Marsalek's biotypes E and F) are currently classified as *S. intermedius*.[50] Separation of the types of *S. aureus* found on humans and domestic animals is shown in Table 47–3.

47.3 CHARACTERISTICS OF DISEASE

Staphylococcus aureus is an opportunistic pathogen causing a wide range of diseases and intoxica-

Table 47–3 Subdivision of *Staphylococcus aureus* into Biotypes/Ecotypes

	Biotypes/Ecotypes			
	A	*B*	*C*	*D*
Staphylokinase	+	–	–	–
Coagulation of:				
Human plasma	+	+	+	+
Bovine plasma	–	–	+	–
Thermonuclease (TNase)	+	W	+	+
Alpha hemolysin	+	V	V	–
Beta hemolysin	V	–	+	+
Origin	Human	Pig/ poultry	Cattle/ sheep*	Hares

*Cattle and sheep strains can be separated by the characteristics of their colonies when grown on crystal violet agar.

W:weak; V:variable.

Source: Adapted with permission from V. Hajak and E. Marsalek, The Differentiation of Pathogenic Staphylococci and a Suggestion for Their Classification, *Zentralblatt fur Bakteriologie, Mikrobiologie, und Hygiene, 1 Abt. Originale*, Vol. 217, pp. 176–182, © 1971, Urban & Fischer.

tions in warm-blooded animals. These include skin infections such as ulcers, carbuncles, impetigo, and epidermidis necrosis (scalded skin syndrome); infections of the internal organs such as bacteremia, endocarditis, pericarditis, osteomyelitis, meningitis, and mastitis; and intoxications such as toxic shock syndrome and food poisoning.[27,55]

Staphylococcal food poisoning is caused by staphylococci (principally *S. aureus*) growing in food and forming enterotoxins as a product of their metabolism. The symptoms of poisoning include nausea, vomiting, abdominal cramps, and diarrhea. These first occur between 1 and 8 hours (usually 2 to 4 hours) after consumption of a contaminated food. In severe cases, collapse may occur but recovery usually is rapid (within 2 days) and uneventful.

While staphylococcal food poisoning is almost always caused by *S. aureus*, enterotoxins also may be produced by some strains of *S. intermedius, S. hyicus, and S. chromogenes*;[2,25,64] these and some other coagulase-negative staphylococci may cause typical staphylococcal food poisoning.[29,36,158]

47.4 MECHANISM OF PATHOGENICITY

Staphylococci produce a wide range of toxins and exoenzymes including membrane-damaging toxins (alpha, beta, gamma, and delta toxin and leucocidin), epidermolytic toxin, and enterotoxin.[6] Staphylococcal enterotoxins (SEs) are low molecular weight (26 to 30 kDa), single chain proteins with isoelectric points between 5.7 and 8.6.[29,171] Their molecular composition is characterized by containing only two residues of half cystine and one or two residues of tryptophan; they contain relatively large amounts of lysine, tyrosine, aspartic, and glutamic acid.[29] They are markedly resistant to degradation by proteolyic enzymes and heat (see Section 47.7.1).

Currently, 10 enterotoxins are recognized: SEA, SEB, SEC$_1$, SEC$_2$, SEC$_3$, SED, SEE, SEF, SEG, and SEH.[27,171] Enterotoxins A, D, and E have very similar molecular compositions, as have SEB, SEC$_1$, SEC$_2$, and SEC$_3$; SEF, SEG, and SEH have dissimilar compositions.[29,171]

The locations of the genetic material responsible for control of a number of these enterotoxins have been identified. The genes responsible for the production of SEA, SEB, and SEC are on the chromosome, whereas those of SED are on a plasmid;[29,88] the nucleotide sequences of the genes responsible for the main enterotoxins also have been identified.[31,155] Some enterotoxins, such as SEA and SED, are produced and released by the cell throughout the growth phase, whereas others such as SEB and SEC are typical secondary metabolites and are produced by the cell at the end of the growth phase and the beginning of the stationary phase.[28,29] This difference also is reflected by the amounts of different enterotoxins produced by *S. aureus* growing under optimal conditions in laboratory media. These may exceed 100 µg ml^{-1} for SEB and SEC production, compared with only 1 to 10 µg ml^{-1} for SEA and SED production.[155] More importantly from the point of view of their production in foods is that SEA and SED (the principal toxins involved in staphylococcal food poisoning in man) are produced in foods under a wider range of pH, E_h, and a_w than are the other enterotoxins (see Section 47.7).

Staphylococcal enterotoxins and the closely related toxic shock syndrome toxin are of particular interest to immunobiologists as they are "super antigens" and are able to bind to the major histocompatability complex (MHC) class II antigen, causing massive T cell stimulation with the release of cytokines.[41,70,145] The ability of SEs to bind to MHC class II molecules and cause the release of cytokines has been suggested by some workers to be the mechanism whereby enterotoxins cause the vomiting response typically following the ingestion of these proteins.[70,90] Thus, the release of cytokines may result in stimulation of neuroreceptors in the intestinal tract, and triggering of the vomiting center in the brain. However, this mechanism of action has yet to be proved, and many studies aimed at understanding the mechanism of action of SEs have not provided experimental evidence to support hypotheses of their mode of action.[62]

The amount of toxin necessary to cause illness depends on the sensitivity of the individual. Factors affecting sensitivity include weight and immunological status; animals exposed to enterotoxins rapidly become resistant to the specific enterotoxin fed or injected intraperitoneally, and sensitization to enterotoxin occurs among people exposed to the toxin in the laboratory. In a study in which toxin was fed in varying amounts to human volunteers, an emetic dose (ED_{50}) of 0.2 $\mu g\ kg^{-1}$ was obtained for SEA, SEB, and SEC equivalent to an amount of 10 to 20 μg for an adult. However, there is much evidence, based on the measurement of amounts of SE in foods implicated in food poisoning incidents, that the amount able to cause illness may be much smaller and it is generally accepted that 1 μg of SE can cause food poisoning.[57,127,168] To produce sufficient enterotoxin to cause illness, a producing strain needs to produce a concentration of cells of at least 1 million g^{-1} or ml^{-1}.

47.5 INCIDENCE OF THE ORGANISM IN THE ENVIRONMENT AND IN FOOD

Staphylococcus aureus occurs widely as a commensal on the skin and mucous membranes of warm-blooded animals. Frequency of occurrence on human skin is rather variable but numbers occurring on normal, undamaged, and disease-free skin generally are low. The organism is transient and does not usually become part of the resident flora of the skin. However, under conditions where the balance of the normal microbial flora is disturbed, for instance by the topical application of a broad-spectrum antibiotic or frequent immersion of hands in water resulting in soreness and damage to the skin, *S. aureus* may become established as part of the resident flora and multiply to high numbers. The latter conditions have been known to occur on the hands of nurses in hospitals and operatives in food-processing plants, eg, among those stripping cooked meat from chicken carcasses, and result in extensive carriage of *S. aureus* on the hands. More usually, *S. aureus* is carried in the nares and perineum and will be present in these regions of the body in about 50% of the population. It will be found in similar sites in other animals; nares being a particular source.

Determinations of numbers on the skin of food animals indicate a generally high incidence but, in the absence of disease, only low numbers. For instance, numbers on the skin of healthy poultry are generally <100 g^{-1} but numbers may be several orders of magnitude higher on processed poultry carcasses as a result of contamination by equipment used in the defeathering process.[104] *S. aureus* also occurs only at low numbers (<100 ml^{-1}) in raw bulk milk but numbers may exceed 1 million ml^{-1} in milk from a mastitic cow.[67] Its wide occurrence in humans and food animals is reflected by its common occurrence in low numbers in many foodstuffs. For instance, in raw beef, 16%;[150] pork sausage, 33%;[58] raw chicken, 41% to 84%;[99,104,150] seafood, 38%;[1] and bakery products, 10%.[147]

Staphylococcus aureus survives well in the environment and is commonly found in food factories where it may become part of the flora of processing equipment and act as a source of contamination or recontamination. This is well demonstrated by a number of studies of the establishment of "endemic" strains of *S. aureus* on the equipment in poultry plants.[103,111] These strains may be an important source of contamination of processed poultry and some may show increased resistance to chlorine.[33] *S. aureus* survives well in dust, and may be found in dust from cyclones and ventilation. Its ability to survive well in the environment is a source of many cross-infection problems in hospitals and has been a common cause of infections among machine operators in engineering plants as a result of multiplication in the fluids used for cooling the metal during milling.

47.6 EPIDEMIOLOGY

Staphylococcal food poisoning mainly is caused by human strains of *S. aureus* (principally Hajek and Marselek's Biotype A) producing SEA and/or D, with the majority of strains producing SEA alone.[84,105,160] While reports of the incidence of enterotoxin-producing strains among strains of *S. aureus* isolated from humans and other animal species show wide differences,[49] the incidence of enterotoxin-producers among human isolates generally is reported to be much higher than among nonhuman isolates. Thus, the incidence among human isolates is reported to vary from 60% to 80%,[105,126] which contrasts with incidences of 3% to 50% for strains isolated from animal sources.[49,137]

A further difference between enterotoxin production by strains from human and nonhuman sources is that the latter more frequently produce enterotoxins other than Type A, with SEC and SED being the most commonly occurring types.[49,67,84] For instance, in a study of the incidence of enterotoxin-producers among Hajek and Marsalek's biotypes of *S. aureus*, isolated from man and domestic animals, 75% of

Biotype A strains from humans were SEA, SEB, or SEC producers (as were 28% of the human Biotype A isolated from domestic animals), while only 1.2% of 490 isolates from other animals (all but one belonging to Biotypes B and F) produced these enterotoxins;[72] SEC production is common among coagulase-negative staphylococci producing enterotoxins.[158]

47.6.1 Incidence of Outbreaks and Sporadic Cases

The reported incidence of staphylococcal food poisoning in Western countries generally has decreased numerically over the past 25 years. It was the most frequently reported cause of foodborne illness in the United States (US) in the early 1970s and was responsible for 29% of the reported outbreaks and 39% of the cases;[34] this compared with 9% of outbreaks (6% of cases) reported to be caused by salmonella. This contrasts with an incidence of 2.0% of outbreaks (10% of cases) reported in England and Wales for the same period with the majority of outbreaks (98%) caused by salmonella.[76] A large part of this difference probably reflects the status of the reporting systems in these two countries at that time, and these differences largely have vanished over the past two decades. Thus, the number of outbreaks of staphylococcal food poisoning reported in the US in 1983–1984 was approximately 10% of the total, with the majority of the reported outbreaks and cases caused by salmonella;[154] however, staphylococcal food poisoning remains the main reported cause of food poisoning in a number of countries, eg, Brazil and Egypt.

The incidence of staphylococcal food poisoning reported for 22 European countries for 1990-1992 ranged from approximately 20% to <1% of total outbreaks with an overall figure of 3.5%;[165] the United Kingdom (UK) reported one of the lowest incidences of outbreaks of staphylococcal food poisoning (<2.0% of the total).

The size of most staphylococcal food poisoning incidents is quite small (10 cases or less). The most frequent cause of these incidents is the preparation of cooked food well in advance of consumption, followed by poor handling and storage. Large incidents have involved food preprepared for a party or an outside event and where the food has been handled by an *S. aureus* carrier during preparation, followed by the food being kept at warm ambient temperatures for 4 or more hours prior to consumption. Foods involved have included cooked meats, poultry, seafoods, and cream-filled bakery products.

Although not common, incidents involving manufactured foods do occur and may involve 100 or more ill people. Reported incidents include:

- cheese, in which failure of a starter culture allowed *S. aureus* to grow to high numbers and to produce enterotoxin;[172]
- salamis, in which production conditions were changed—ie, fermentation temperature raised to speed up the manufacturing process—allowed *S. aureus* to grow to sufficient numbers to produce enterotoxin before growth was suppressed by acid production by the fermentation flora;[113,157]
- pasta, in which *S. aureus*-contaminated egg used in a pasta mix grew to high numbers in the mix during the extended blending and drying processes at 30° C to 40° C;[168]
- canned meat, fish, and vegetable products contaminated after the sterilization processes as a result of manually handling warm and wet cans or contact of the seams of such cans with equipment contaminated with *S. aureus*;[18]
- bakery products contaminated during decoration or filling.[147]

47.7 FACTORS AFFECTING GROWTH AND SURVIVAL OF *S. AUREUS* AND PRODUCTION AND DESTRUCTION OF ENTEROTOXIN

47.7.1 Temperature

Growth. Most strains of *S. aureus* will grow aerobically at temperatures between 7° C and 48° C and have an optimum growth temperature of 35° C to 40° C. Generation times in foodstuffs stored aerobically at 35° C to 40° C can be as short as 20 min, with times increasing to 1 to 2 hours at 20° C, 5 to 10 hours at 15° C and 12 to 24 hours+ at 10° C.[82] Growth rates in vacuum- and gas-packed foods are usually significantly slower than in air-packed foods, the specific growth rates depending on the product composition, storage, and packaging conditions.[22,75,100] Lag times (times to the commencement of growth) are similarly affected by temperature and may vary from 1 to 7+ days at 10° C to 2 to 3 hours at 35° C to 40° C.[82] Lag times are particularly affected by the previous history of the cell; thus, lag times for cells damaged by harmful conditions such as freezing, drying, or heat will be variable, but much longer than the times mentioned above. After growth commences, the previous history of the cell does not affect the growth rate or the amount or rate of enterotoxin production.

Survival. *S. aureus* survives freezing well, and at –18° C will survive in meat and poultry for at least 6 months with little or no change in cell numbers;[164]

however, significant kill will occur at temperatures of –10° C and above in some substrates.[167]

Staphylococcus aureus has a heat sensitivity typical of most Gram-positive bacteria, ie, a *D* value of 1 to 6 minutes at 60° C (z = 6° C to 10° C) when heated in high a_w foods such as milk, meat, and vegetables;[11,56,60] $D_{60° C}$ values in phosphate buffer (pH 6.5 to 7.5) range from 1 to 2.5 minutes.[166] As would be expected, cells become much more resistant to heat when heated in a low a_w food. Thus, a $D_{60° C}$ value in milk of 5.3 minutes was increased to 42.3 minutes in milk containing 57% sucrose,[87] and a *D* value of 3 minutes in an egg pasta mix of a_w 0.92 was increased to a $D_{60° C}$ value of >40 minutes in an egg pasta mix adjusted to an a_w of 0.80.[78] Also the $D_{60° C}$ value in a meat product containing 3% to 4% NaCl was increased from 6 to 25 minutes to when the salt content was raised to 8.4%.[19] Lowering or raising the pH from neutrality reduces the heat resistance.[146] Cells of *S. aureus* heated in oil or fat can show remarkably high heat resistance (ie, a $D_{120° C}$ value of 3 to 6 minutes; z = 33° C);[65] however, the presence of small amounts of moisture significantly reduces this heat resistance.[170]

Other factors that will affect the measured heat resistance of *S. aureus* include: the temperature at which cells are grown, ie, cells grown below 30° C are much less resistant to heat than cells grown at 37° C, whereas those grown above 40° C are more resistant;[142] age of cells, ie, early log phase cells of *S. aureus* are about one-third as resistant to heat as stationary phase cells;[159] recovery conditions—the optimum temperature and pH for the recovery of heat-damaged cells of *S. aureus* are 32° C and pH 6.0.[5]

Heating causes damage to the cell systems that produce catalase and protect the staphylococcal cell from peroxides. The addition of blood or pyruvate to the recovery medium protects the cell while recovering from this heat-induced damage, allowing the recovery of most of the heat-damaged cells.[15,61]

Enterotoxin Production. Enterotoxins are produced under a narrower range of temperatures than those permitting growth, with temperature affecting both the amount and rate of production; the optimum temperature for enterotoxin production is the same as that for growth. There is extensive literature on the effect of temperature on enterotoxin production in foods;[82] however, because of the different numbers of organisms used as inocula, the effect of different microfloras (qualitative and quantitative) on growth and enterotoxin formation, and the very different sensitivity of methods used to detect whether enterotoxins have been formed, it is difficult to compare results obtained in one series of experiments with another. In one study, sufficient enterotoxin (SEA) to cause illness was produced in pasteurized milk with an initial microflora of 10^4 ml^{-1} stored for 6 hours at 35° C, 18 hours at 25° C, and 36 hours at 20° C, whereas in milk with an initial microflora of 10^6 ml^{-1}, these times were doubled, and in milk with an initial microflora of >10^6 ml^{-1}, SEA was detected only in the milk stored at 37° C.[54]

The limiting temperature for enterotoxin production is about 10° C. At this temperature, the rate of enterotoxin is very slow and in one experiment, 4 weeks were needed to produce detectable SEB from an inoculum of 10^6 g^{-1} in ham.[66] With a similar size of inoculum in brain heart infusion broth, using a more sensitive enterotoxin detection system, SEA was detected after 2 weeks at 10.5° C but not after 4 weeks at 9.3° C.[133] The maximum temperature for enterotoxin production is 45° C to 46° C, but this limit can be raised by several degrees by growth in the presence of 1M sodium chloride.[79] At suboptimal growth temperatures, the concentration of cells needed to produce sufficient enterotoxin to cause illness is increased, and a concentration of cells of approximately 10 million g^{-1} or ml^{-1} is required at temperatures of 20° C to 25° C.

Enterotoxin Destruction. Staphylococcal enterotoxins are resistant to freezing and destruction by heat. They will survive all types of commercial pasteurization processes and may even survive some used for the sterilization of canned foods.[23] In whole milk, enterotoxin survived heating for 20 minutes at 121° C ($D_{120° C}$ = *ca.* 20 minutes), and based on heat destruction, data at lower temperatures were estimated to have a z value of 29° C.[149] In a cream of vegetable soup (initial concentration 2.5 µg ml^{-1}), SEA survived a thermal process of f_0 = 3.0 minutes;[23] heat resistance is significantly reduced at acid pH values.[149] Heat may result in the loss of antigenicity of enterotoxins before loss of biological activity. Urea treatment of extracts from heat-treated foods, prior to use of immunological methods for enterotoxin testing, has been used as a means of restoring antigenicity.[23,24]

47.7.2 Water Activity

Growth and Survival. *Staphylococcus aureus* is one of the most tolerant foodborne illness-causing bacteria to low water activity and grows at a_ws well below those of other foodborne pathogenic bacteria.

Optimal survival is at a_ws of 0.07 to 0.16.[134] In dust, death rates increase at a nonuniform rate with increase in relative humidity (RH), reaching a maximum at an RH of 50% to 60%; survival rate under dry conditions decreases with increased temperature.[98] Death rate under dry conditions also is affected markedly by the availability of air, and Rahn concluded that the death rate of *S. aureus* is proportional to the square root of the oxygen concentration.[124] The adverse effect of air on the survival of *S. aureus* under dry conditions can be illustrated by experiments in which cells were inoculated into dry foods adjusted to different a_ws and stored in air and under vacuum at 25° C and numbers of survivors counted during storage. In a cake mix (a_w 0.53), the time for a 99.9% decrease in count was 8 weeks in the air-stored cake mix but 18 weeks in the vacuum-stored product; at a_ws of 0.00 to 0.22, there was virtually no change in the initial inoculum count during 27-week storage of the vacuum-stored cake mix, and only a 90% decrease in numbers in the air-stored mix.[42]

The limiting a_w for growth depends mainly on humectant, atmosphere, pH, and temperature. The limiting a_w for the growth of *S. aureus* in brain heart infusion broth (pH 7.0) incubated aerobically at 30° C was 0.86 in the presence of sodium chloride and 0.89 in the presence of glycerol;[101] the optimum a_w for growth in the presence of sodium chloride was 0.97 (4.0% NaCl).[80]

The interacting effects of temperature, atmosphere, and pH on the limiting a_w for the growth of *S. aureus* are illustrated in Table 47–4.

This table shows that the limiting a_w for growth is lower under aerobic than anaerobic conditions and that strains producing enterotoxins A, B, and C have similar growth limits; lowering temperature and pH gave expected increases in the limiting a_w for growth.

Enterotoxin Production. Table 47–4 also shows the effect of a_w on the production of SEA, SEB, and SEC; SEA was produced at much lower a_ws than SEB or SEC under all conditions of growth. The limiting a_w for the production of detectable enterotoxins (detection limit 50 ng ml^{-1}) was significantly higher than that limiting growth.

47.7.3 pH

Growth and Survival. The range of pH values at which *S. aureus* is able to grow is approximately 4.0 to 10.0 under otherwise optimal growth conditions; the optimum pH for growth is pH 6.0 to 7.0. At the extremes of pH, growth is very slow. Thus in UHT milk adjusted to acid pH values by the addition of HCl, the lag and generation times at pH 4.3 were respectively 2.8 days and 1 day at 10° C, 2.7 days and 14.4 h at 13° C, and 11 h and 2.6 h at 20° C.[82]

Organic acids at equivalent pH values to those obtained using inorganic acids are, as would be expected, more effective against *S. aureus*. Survival times in the presence of acetic acid depend on temperature; in mayonnaise at pH 3.9 (0.39% acetic acid), the survival time of an inoculum of 10^7 cells ml^{-1} was 1 day at 30° C and 9 days at 10° C;[163] 1% acetic acid at pH 5.0 also is bactericidal.[3] At equivalent concentrations of undissociated acid, lactic and citric acids are less effective than acetic acid against *S. aureus*; citric acid is used by the cell as a carbon source.

Enterotoxin Production. Staphylococcal enterotoxins are produced under a more limited range of pH values than those allowing growth, and the practical limit in acid foodstuffs is pH 5.0. The reported optimum pH values for the production of enterotoxins suggest that this lies between pH values 7.0 and 8.0. Staphylococcal enterotoxin A is produced under a wider range of pH values than is enterotoxin B.[130] For example, SEA was produced in mayonnaise adjusted to pH 5.1 and stored at 37° C, whereas SEB, SEC, and SED were produced only at higher pH values.[69]

47.7.4 Gases

Staphylococcus aureus grows faster under aerobic than anaerobic conditions and, as noted above, also produces enterotoxins under a wider range of temperatures and pH values when grown aerobically (Table 47–4). However, survival is better under anaerobic conditions. Thus in a series of experiments on the growth of *S. aureus* in nitrogen- and air-packed sandwiches stored at 26° C, growth rates were higher in the air-packed sandwiches, and enterotoxins were produced much faster in the latter; enterotoxins A and D also were detected much earlier than enterotoxin B.[22] Carbon dioxide (75%) was inhibitory to growth in sliced cooked beef at 12.8° C.[75]

47.7.5 Preservatives

Growth and Survival. *Staphylococcus aureus* is sensitive to sorbic acid, and in a model meat product with pH values 5.4 and 5.7, 5000 ppm was bactericidal.[143] In the same product, 1000 ppm of a mixture of 3:1 w/w sorbic acid:glycerol monolaurate was inhibitory in product with a pH value

Table 47–4 Limiting Water Activities for Growth and Enterotoxin* Production by Cocktails of Strains of S. *Aureus* Grown under Aerobic and Anaerobic Conditions in Brain Heart Infusion Broth at 37° C and 22° C[12]

	pH 6.8				*pH 5.0*			
	Aerobic		*Anaerobic*		*Aerobic*		*Anaerobic*	
	37° C	22° C	37° C	22° C	37° C	22° C	37° C	22° C
Growth	0.86	0.90	0.88	0.90	0.90	0.94	0.92	0.94
SEA	0.90	0.92	0.90	0.94	0.92	0.96	0.94	0.96
Growth	0.88	0.90	0.88	0.90	0.90	0.94	0.90	0.94
SEB	0.92	0.96	0.95	0.96	0.96	0.98	0.98	0.98
Growth	0.88	0.90	0.88	0.90	0.90	0.94	0.92	0.94
SEC	0.96	0.96	0.96	0.96	0.98	0.98	0.98	0.98

* limit of enterotoxin detection: 50 ng ml^{-1}.

Source: Adapted with permission from A.C. Baird-Parker, The Staphylococci—An Introduction, *Journal of Applied Bacteriology, Supplement,* Vol. 69, pp. 1S–8S, © 1990, Blackwell Science, Ltd.

of 5.4, and stored anaerobically at 35° C; 200 ppm sodium nitrite was inhibitory to anaerobic growth at pH 5.4 and aerobic growth at pH 5.7.[143] Nitrite is more inhibitory to growth under anaerobic than aerobic conditions.[92]

S. aureus will grow in the presence of >20% NaCl under optimal growth conditions, although in foods stored at temperatures <20° C, 15% will prevent growth. The inhibitory effect of NaCl is increased markedly in the presence of the antioxidant butylated hydroxyanisole (BHA). Thus, in the presence of 100 ppm BHA, 3% NaCl was inhibitory to growth in trypticase soy broth (pH 6.0) at 37° C and under the same conditions with 7% NaCl, 50 ppm BHA was inhibitory.[144] Ethanol at concentrations above 7% is inhibitory to *S. aureus* and 9% or more is bactericidal.[112,135]

Enterotoxin Production. With the exception of information on the effect of curing salts (see Section 47.7.2), there is little information on the effect of preservatives on enterotoxin production. The small amount of information available indicates that they have no specific effect on enterotoxin production other than what can be explained by their effect on growth. Thus, the limiting concentration of a preservative, such as sorbic acid, on enterotoxin production is close to the minimum inhibitors concentration (MIC) value for growth. However, in one series of experiments, ethyl-4-hydroxybenzoate had a pronounced effect on enterotoxin production and prevented production at concentrations that had no effect on growth.[82]

47.7.6 Irradiation

Staphylococcus aureus is sensitive to ionizing irradiation, and *D* values between 0.2 and 0.4 kGy have been reported in a variety of meat and fish products.[122,123,139] Staphylococcal enterotoxins are extremely resistant to ionizing irradiation and are unaffected by sterilizing doses.

In phosphate buffer (pH 6.8) a dose of 100 Kergs mm^{-2} of ultraviolet light (wavelength 250 to 280 nm) gave a 2 log kill of *S. aureus* at –79° C, whereas at 22° C a dose of 500 Kerg mm^{-2} was required for this kill; the difference was that at –79° C, there was no shoulder on the survival curve.[7]

47.7.7 Disinfectants

Staphylococcus aureus is sensitive to the wide range of disinfectants used by the food industry. Typical values at ambient temperatures (15° C to 30° C) for destruction in the absence of protein are: hypochlorite (10 ppm), 5 log kill in 30 seconds; quaternary ammonium compound (QAC) (30 ppm), 5 log in 30 seconds; Iodophor (10ppm), 5 log in 30 seconds; peracetic acid (200 ppm), 5 log kill in 1 minute; hydrogen peroxide (300 ppm), 5 log kill in 5 minutes; phenolic household disinfectant (10,000 ppm), 5 log kill in 10 minutes.[85,86,108] In the presence of protein, the amounts of these disinfectants to give equivalent kills will be significantly higher. For instance, in a dry milk film, 200 ppm of chlorine and a contact time of 2.5 minutes were found to be necessary for a 5 log kill[109] and the presence of even small amounts of protein, ie, 0.5% skim milk, resulted in more than doubling of the amounts of QAC, iodophor, and chlorine required to give a 5 log kill.[86]

47.7.8 Competitive Microorganisms

Staphylococcus aureus is a poor competitor and growth is readily prevented by other microorganisms. This is well illustrated by poor growth in raw meat but excellent growth in cooked meats. The reasons for such poor growth are variously described to be the result of: the production of organic acids, resulting in the lowering of pH; the utilization of oxygen by competitor microorganisms, resulting in the reduction in E_h and anaerobiosis; depletion of a specific nutrient; and the production of specific antimicrobial factors.[113] All or some of the factors may play a role in some foods, but the most likely causes of inhibition in foodstuffs are pH drop and anaerobiosis. The production of antimicrobial substances against *S. aureus* has been claimed for a wide range of bacteria including pseudomonads, enterobacteriaceae, bacillaceae, and fecal streptococci, these organisms affecting growth as well as enterotoxin production.[71,102,110] However, the production of specific antimicrobial substances was not demonstrated by these workers and, although a possible role of bacteriocins and other antimicrobial substances (such as hydrogen peroxide) cannot be ruled out, there is little evidence to support their role in inhibiting *S. aureus* in foodstuffs.

47.7.9 Interactions

In most foodstuffs, no single factor controls the growth, survival, or enterotoxin production by *S. aureus*, but rather a combination of factors such as reduced a_w, pH, E_h, and temperature.[141] Such interactions have been studied in most detail by those concerned with the mathematical modeling of microbial growth or survival, where the aim has been to develop models able to predict the fate of an organism in a foodstuff subjected to a particular process, formulated in a particular way, and/or stored under certain conditions.[148] One of the best illustrations of the effect of interacting factors on *S. aureus*, and its ability to produce enterotoxin in a foodstuff, is to consider the production of a traditional fermented, dry salami characterized by Genoa and Hungarian salamis. The principal factors preventing the growth of *S. aureus* in such salamis are shown in Exhibit 47–1; see also Chapter 19.

Exhibit 47–1 Factors Affecting Growth and Toxin Production of *Staphylococcus aureus* in Fermented Sausages[112]

Abiotic Factors

1. Physio-chemical factors: pH, E_h, and a_w.
2. Additives: type and amount of carbohydrate; nitrites/nitrates; glucono-D-lactone; NaCl; spices.
3. Conditions during sausage manufacture: Oxygen tension; temperature; humidity.

Biotic Factors

1. Number of *S. aureus* cells initially present in meat.
2. Use of starter culture: type and inoculum size.
3. Numbers and types of competitive organisms present.

Source: Adapted with permission from G.J.E. Nychas and J.S. Arkoudelos, Staphylococci: Their Role in Fermented Sausages, *Journal of Applied Bacteriology, Supplement*, Vol. 69, pp. 167S–188S, © 1990, Blackwell Science, Ltd.

These factors include selection of meats and other ingredients used in sausage manufacture that are free from or only contain low numbers of *S. aureus*; promoting a rapid drop of pH to <5.3 by use of a suitable starter culture, combined with an appropriate fermentation temperature; and drying under conditions of controlled humidity and temperature to promote thorough drying throughout the sausage to the predetermined water content. Measurements of the fate of *S. aureus* during the salami manufacturing process show the tendency for better growth of *S. aureus* at the surface of salamis fermented in the presence of air, with the production of enterotoxin only in the surface layers; the growth of *S. aureus* at the surface of sausages fermented in brine is inhibited, an effect claimed to be the result of the greater inhibitory effect of nitrite under anaerobic storage conditions.[92] The use of modified atmosphere packaging during the ripening of salamis has been suggested as a means of reducing the possibility of enterotoxin production.[92,141]

47.8 PRINCIPLES OF DETECTION AND ENUMERATION

47.8.1 Detection of *S. aureus*

Isolation. Many methods have been described for isolating *S. aureus* from foods and other sources. The productivity of these methods has been reviewed and assessed by many workers.[9,125,151] The solid (agar) media that may be used for the isolation and enumeration of *S. aureus* in foods can be divided into three broad types.

- The first, and most simple of these media, uses sodium chloride as the selective agent and incorporates a metabolizable substrate such as mannitol (production of acid), blood (hemolysis), or milk (zones of clearing/precipitation) as diagnostic agents.[16,81] These media have three main problems: they lack specificity (there are many salt-tolerant organisms other than *S. aureus* in foods); the substrates used to indicate the presence of *S. aureus* also are metabolized by other organisms; and cells of *S. aureus* when subjected to stress, by, for instance heating, drying, or freezing, become sensitive to NaCl and will not be recovered on salt-containing media unless they are first allowed to recover in a salt-free medium.[15,81]
- The second group of media contain a combination of substances intended to achieve good selectivity, with egg yolk as the diagnostic agent. *S. aureus* uses the lipoprotein lipovitellenin, contained in egg yolk,[136] resulting in the formation of clear zones around colonies of *S. aureus* growing on an egg-yolk–containing agar medium; this may be followed, on further incubation, by the formation of a white precipitate within the clear zones as a result of precipitation of calcium and magnesium salts of fatty acids released by the hydrolysis of fat.[153] The types of selective agents used in this group of media include antibiotics, such as polymyxin and sulphamezathine, and inorganic salts such as lithium chloride, potassium thiocyanate, and potassium tellurite. The latter compound is metabolized by *S. aureus* with the release of tellurium, resulting in the formation of black colonies, which is a further useful diagnostic feature on tellurite-containing media. Examples of this second group of media are tellurite polymyxin agar,[45] KRANEP agar,[138] and Baird-Parker agar.[16] The latter medium has been subjected to many international trials, demonstrating a generally superior performance over other media and thus remains the medium of choice for the enumeration of *S. aureus* in foodstuffs.[8,9,37,97,151] The good performance of this medium depends on: a balance of selective agents (lithium chloride, glycine, and potassium tellurite) to inhibit Gram-negative and Gram-positive bacteria other than *S. aureus*; the inclusion of sodium pyruvate to protect *S. aureus* cells damaged by heat from being killed by peroxides and other oxidizing agents (such damaged cells lose the ability to produce catalase); and the presence of egg yolk, which, in addition to providing one of the main diagnostic features of the medium, also assists in the recovery of damaged cells.

  There are a number of variants of Baird-Parker's medium intended for particular situations. The addition of sulphamezathine is recommended for the detection of *S. aureus* in raw meat and fish products, where overgrowth by *Proteus* spp. may occur,[140] and for generally heavily contaminated products, the further addition of acriflavine and polymyxin is recommended.[52] The prolonged storage of prepared plates of Baird-Parker agar may result in loss of selectivity, so if plates are intended to be stored for more than 48 h, it is recommended that the stable (pyruvate-free version of the medium) be used, the pyruvate being spread onto the medium immediately before use.[77] Good descriptions of the preparation and use of Baird-Parker agar will be found in the following references.[37,81,97]
- A third group of media has been devised to overcome one of the principal drawbacks to the use of egg-yolk–containing media for the detection of *S. aureus* in foodstuffs, namely that some strains from animal sources (principally from cattle), do not use lipovitellenin as a substrate and hence colonies do not show clearing on egg-yolk–containing agars.[14,91] This problem is well-recognized by those using the medium for the examination of dairy products, and they rely mostly on the typical morphology and color of colonies of *S. aureus* on Baird-Parker agar, as indicators of *S. aureus* presence. However, the need for additional testing to confirm *S. aureus* is a limitation to the use of this medium and this has led to the development of Baird-Parker medium containing pig plasma[83] or rabbit plasma with bovine fibrinogen[21] in place of egg yolk. The diagnostic feature is thus the coagulation of the plasma, resulting in a zone of precipitation around and beneath a positive colony. These methods continue to show promise, but because of cost and variable performance of commercially available plasmas, they are not used widely. The use of the egg-yolk–containing Baird-Parker medium continues to be the preferred method.

The use of solid media for the detection and enumeration of *S. aureus* in foods may be limited to the amount of food that can be spread on a plate. In practice, no more than 0.5 to 1.0 ml of a 10% suspension of a food can be spread on a standard agar plate without overloading the plate with particles of food, making the plates difficult to read; this amount can be increased to 1 to 2 ml on large size Petri dishes. With the normal dilution of a food (1 in 10), the limit of detection is 10 to 20 cells ml^{-1} or g^{-1} of food, unless multiple or large plates are used. This is adequate for most purposes, but if the detection of less than 10 cells is required, a most probable number (MPN) tube method should be used. For this purpose, the medium of Giolitti and Cantoni[68] with the addition of Tween 80[81] is recommended. Confirmation of the isolation of *S. aureus* is obtained by plating out positive (black) enrichments onto Baird-Parker agar or some other selective and diagnostic medium. More recently, the US Association of Official Analytical Chemists' approval was given to a more simple enrichment broth for MPN determination of *S. aureus*. This is based on the use of trypticase-soy broth containing sodium chloride as the selective agent with the addition of sodium pyruvate to aid the recovery of stressed cells.[96] The use of enrichment broths to isolate low numbers of *S. aureus* from foodstuffs is further discussed by Baird and Lee.[9]

Confirmation. The principal method used to confirm that an isolate is *S. aureus* is to apply the coagulase test. The preferred test format is the tube coagulase test using either human or rabbit blood plasma as the test substrate;[37,81] citrated plasmas should be avoided as the breakdown of citrate by some organisms growing on egg-yolk agars, eg, fecal streptococci, may result in false-positive reactions.[14] If the "slide coagulase test" is used (this will detect the presence of the clumping factor), the organism should be subcultured onto a nonselective medium before carrying out the test.

A variety of rapid and convenient tests for the detection of staphylocoagulases are available commercially; these are mainly based on the slide coagulase or clumping factor test. They include a latex agglutination test that detects the clumping factor and protein A, and plasma-coated red blood cells detecting the clumping factor.[4,39] It should be noted that the production of coagulases is not a property of only *S. aureus*, but other bacteria, including some Gram-negative bacteria and other staphylococci such as *S. hyicus* and *S. intermedius* will also coagulate plasmas. On tellurite-containing media, *S. intermedius* will not be confused as the organism produces gray colonies. *Staphylococcus hyicus* (principally found on pigs) may be confused with *S. aureus*, and for a definitive diagnosis it is advisable to use additional biochemical tests such as the API Staph-Ident system or the VITEK Gram-positive Identification Card; differentiation is also possible using a slide coagulase test as *S. hyicus* does not produce the clumping factor.

The production of Protein A[40] or TNase[35,93–95] by *S. aureus* is used by some workers as a diagnostic feature, although the use of these properties as confirmation tests would not appear to have any advantage over the coagulase test. However, the TNase test has a useful role in food microbiology because it will survive conditions, eg, pasteurization, that would destroy *S. aureus* but not the enterotoxins. Thus it can be used as a screening test for possible presence of enterotoxins; the microslide method is particularly useful for this purpose.[95]

Future methods for the detection of *S. aureus* in foods and other materials will probably include tests based on the detection of 16S and 23S rRNA sequences or mRNA using reverse transcriptase and DNA probes.[26] Nucleic acid-based probes can also be used as confirmation tests for *S. aureus*.[63,166]

47.8.2 Detection of Staphylococcal Enterotoxins

The traditional use of animal tests for the detection of staphylococcal enterotoxins has been replaced by sensitive immunological methods for their detection and identification.[28,29] The sensitivity, reliability, and convenience of these methods have continued to improve with the advancements made in antigen-detection systems.

The methods used in the 1960s were based on the classic Ouchterlony double-diffusion plate method with the microslide version of the technique being the preferred version of the test as it was more economical on reagents;[38] however, this method was relatively insensitive (limit of detection was ~ 0.5 $\mu g\ ml^{-1}$ of food extract). To be able to detect enterotoxins at concentrations able to potentially cause illness, ie, 0.01 $\mu g\ ml^{-1}$, lengthy extraction and concentration procedures taking 4 to 5 days to complete were required. The need for these extraction and concentration procedures was largely removed by the development of more sensitive antigen detection techniques. The first of these used radioimmunoassay procedures[44] and were not therefore suitable for wide-scale use by the food industry. The development of enzyme immunoassay (EIA) methods for enterotoxin detection and typing and in particular enzyme-linked immunosorbent assay (ELISA) methods[59,121] and the reverse-passive latex agglutination test[129] has led to the widespread availability of sensitive and convenient assay methods. These methods could only be used as suitable polyvalent and more recently monovalent antisera became available and this depended on the application of modern protein purification techniques by Bergdoll and others to purify the SEs and to use these to make high-quality antisera.[27,155] Methods of SE detection now are available commercially and have reduced preparation and detection time from several days to a few hours. The following is a description of the performance and use of four of the most widely used, currently available test kits.

- **Bommeli Kit** (Bommeli, Switzerland). This is an EIA kit based on the use of monovalent antibodies to SEA to E and was one of the first available commercial kits for SE detection. The specific antibodies are attached to plastic balls, which are used as the enterotoxin capture and detection system. The use of this method for foods requires extraction, taking 1 to 2 days to complete. The method has good sensitivity (0.1 to 10 ng ml^{-1} of food extract) but interference by food components has been reported leading to some false-positive and -negative results.[20]
- **TECRA Kit** (Bioenterprises Pty., Australia). This EIA kit uses polyclonal antibodies to SEA–E as capture antibodies in a standard microtitre format plate. It is widely used, having high sensitivity (1 ng ml^{-1}), speed (results in 4 h), and convenience. The method generally has good reliability but some false-positive results may arise from the presence of peroxides and non-enterotoxin metabolites in foods.[48,118,119,169]
- **VIDAS Kit** (Biomerieux, France). This is one of a series of test kits for use with the automated VIDAS ELISA system. It detects SE A–E, and is very fast (results in 80 min) and sensitive (1 ng ml^{-1}). Versatility and good performance (there are few reports of false-positive and -negative results) has led to wide use by laboratories involved in the frequent examination of foods for enterotoxins.
- **Oxoid Kit** (Oxoid, England). This is an RPLA (reverse-passive latex agglutination) test using monoclonal antibodies for SE capture and detects SE A–D. While it lacks the sensitivity, specificity, and speed of EIA/ELISA test kits, it is simple to use and does not require special equipment for carrying out the test. It does, however, require the use of a concentration step to be applied to a food extract before applying the test, and various food components interfere with the test leading to false-positive and -negative reactions.[117,129]

All methods used to test for SEs in foods appear potentially subject to interference by food components and microbial metabolites, although a recently described kit (RIDASCREEN™), based on the use of monoclonal antibodies to SEA-SEE as capture antibodies, was shown in a recent interlaboratory trial to be fast (results in 3 h), sensitive (1 to 2 ng SE g^{-1}), and highly specific.[120] Also heat and other agents capable of denaturing SEs may interfere with its immunological detection; the use of urea to renature enterotoxin subjected to such denaturation has been proposed.[24]

Despite continued improvement in the performance of commercially available kits for enterotoxin detection, it is strongly recommended that the definitive confirmation of SE in a food, or its absence, should be based on obtaining the same result using at least two different test procedures.

The use of oligonucleotide probes to detect the enterotoxin genes of *S. aureus* has been the subject of a number of recent studies, and probes to all the major SEs have been synthesized;[74] however, it has been questioned by some workers whether such probes are useful for the examination of foods for SEs. There are two potential problems associated with their use. The first is that the probe will only demonstrate the potential to produce SE and therefore can only be used as a screening test for possible presence of enterotoxin; for definitive demonstration of the production of an SE, a direct test for enterotoxin must be used. The second is that *S. aureus* strains that would seem not able to form SEs possess DNA sequences that are silent or mutant copies of the SE gene and probes, therefore, will give a false impression of the potential of a strain to produce enterotoxin.[116]

47.9 CONTROL

Staphylococcus aureus is a ubiquitous organism on warm-blooded animals and its presence in low numbers in raw foods of animal origin is to be expected. Although most strains isolated from animals do not produce enterotoxins, some do, and not infrequently strains of human origin (Biotype A), which are the main enterotoxin producers, will be found on foods of animal origin. It is important, therefore, to avoid the use for further processing of raw materials that may be contaminated with large numbers of *S. aureus*, as although the organism may be killed by such processing, enterotoxins may be present and these are unlikely to be removed or destroyed. Also the handling of such contaminated raw materials in a food processing environment is likely to lead to their establishment in that environment, and/or to become a source of contamination of other products. It is particularly important to control raw materials that are likely to be sources of *S. aureus* if these are to be used in a process, such as raw-milk cheese or fermented sausage manufacture, where further multiplication may occur.

One of the main sources of contamination of foods with *S. aureus* is without doubt the food handler. It is therefore important that all food handlers are made aware of the importance of good personal hygiene and of the need to report any skin infections to management so that the infections may be treated.

Staphylococcus aureus needs appropriate growth conditions to form its enterotoxins. Most foods, and many raw materials and ingredients, will support growth and enterotoxin formation given the right conditions, the most important of which are time and temperature. If such conditions exist in any part of the food chain, it is important to ensure that either the product will not support the growth of *S. aureus* or that the organism is eliminated by appropriate processing and that the product is then protected from potential sources of recontamination. Whereas processing (eg, heating) may eliminate *S. aureus* cells, it will not destroy any enterotoxin formed by growth of the organism prior to processing. Therefore, it is important to avoid significant growth prior to processing.

The application of the Hazard Analysis Critical Control Point (HACCP) to a particular food-processing or food-handling operation is the only certain way to determine whether the presence of *S. aureus* is potentially a hazard in a foodstuff produced under a particular production regime, and to establish what control measures may be needed; see Chapter 58 for the use of HACCP.

47.10 CONCLUDING REMARKS

Despite well-defined methods of control, principally based on the application of good hygienic practice, staphylococcal food poisoning remains a significant cause of foodborne illness in many parts of the world. Recognizing that the source of most strains of *S. aureus* causing gastroenteritis is the human carrier, better education and training of food handlers are likely to have the most impact on such illness.

The heightened medical concerns arising from the increasing occurrence of antibiotic-resistant strains of *S. aureus* does not require food producers to apply additional measures to control these organisms as they are not more resistant than other strains to the sanitizers and other procedures used by the food industry to kill this organism. However, the continued, widespread use of antibiotics during the growing of food animals must inevitably lead to an increased risk of antibiotic-resistant strains of *S. aureus* entering the food chain, infecting humans, and further adding to the already serious clinical problems caused by these organisms.

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48

Vibrio species

Charles A. Kaysner

48.1 Introduction
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48.1 INTRODUCTION

The preponderance of historical information regarding the genus *Vibrio* centers on *Vibrio cholerae* because of the catastrophic events that have occurred as a result of this organism. Prior to the mid 1900s, the organism responsible for cholera was the only human pathogen placed eventually in the taxonomic position that we know today. Since cholera has affected millions of individuals worldwide during the past two centuries, the intensive research of its cause and prevention is more than justified. Only in the last 50 years have most of the other species now included in this genus been identified. This resulted from the recognition that several unidentified organisms were responsible for human illness and also from the environmental occurrence of organisms appearing to be related to *V. cholerae.* Due to the extensive amount of literature regarding the many species of *Vibrios* and due to space constraints, this chapter is a summary of the current information on the more common pathogenic species. This chapter will deal mainly with the major foodborne pathogens *V. cholerae, V. parahaemolyticus,* and *V. vulnificus*, with an emphasis on aspects of major concern to food microbiologists. Detailed reviews of pathogenic vibrios can be found in many studies.[72,119,120,127,168,170]

Man's association with the bacterium now called *Vibrio cholerae* began many centuries ago. Cholera was mentioned in ancient writings nearly 2,500 years ago[110] and was the term given to any type of gastrointestinal disorder, particularly those with resultant diarrhea. The causative agent of cholera was first described in Italy in 1854 by Pacini.[132] He discovered that the intestinal contents of cholera victims contained large numbers of a curved bacterium that he called *Vibrio cholera.* His discovery was overshadowed by that of Robert Koch, who demonstrated while studying the disease in Egypt in 1883 that cholera was caused by a comma-shaped organism. He called the organism "Kommabazillen" and the name *Vibrio comma* was used for several decades. Pacini's work was finally recognized and *Vibrio cholerae,* Pacini 1854, was designated as the type species of the genus. Cholera has afflicted millions of individuals over the centuries and has been one of the most feared bacterial diseases of man, with the exception of the bubonic plague of the 18th century in Europe and Asia Minor. The disease has also been called Asiatic cholera, because the organism subsequently was determined to be endemic in that region and responsible for large epidemics in the Ganges basin of India and Bangladesh. The population of the Bengal region has not been free of the disease in recent times. Cholera is spread rapidly among individuals after drinking water and food have become contaminated because of inadequate sewage disposal and poor sanitation. Cholera epidemics have been associated primarily with underprivileged, low socioeconomic populations lacking potable water sources. Crowded living conditions aid the rapid spread of the disease. Pandemic waves have occurred along trade routes and in association with pilgrimage and migration.

Seven pandemics have been recorded since the early 19th century (Table 48–1). The first six originated on the Indian subcontinent and ended about 1925. The seventh pandemic is notable in that it originated in Indonesia in 1961 and has endured for more than 30 years. This pandemic spread through Southeast Asia, into Asia and the Middle East, and reached the African continent in 1970. Twenty years later cholera reached the South American continent, which had been free of the disease for more than a century. From the initial cases in Peru in January 1991, the epidemic spread rapidly to bordering countries and into Central America. Accumulatively, from January 1991 through June 1994, the Pan American Health Association recorded well over 1 million cases of cholera in 20 South and Central American countries and close to 10,000 deaths.[164] These figures include cases imported into the United States (US) and the United Kingdom (UK). The seventh pandemic is the most notable of those recorded in that it has been the most extensive in geographic spread and in duration.[77]

In the fall of 1992, an explosive epidemic originated in the Bengal region of India from a previously unknown serogroup of *V. cholerae*.[3] All aspects of this epidemic were similar to that caused by the O1 serogroup. This new serogroup was designated O139 and the strain as "Bengal." This epidemic spread through India, eventually to Bangladesh, Nepal, Burma, Pakistan, Thailand, China, Malaysia, and Saudi Arabia. Imported cases also were reported in the UK and the US. The epidemic afflicted thousands of individuals and caused many deaths. The Bengal strain was found to share many virulence features with the El Tor biotype of O1 *V. cholerae* of the seventh pandemic. Because of the similarity of disease, O139 Bengal is considered to be the second etiological agent of cholera. Due to its spread to other countries within a 2-year period, some suggest it is the eighth cholera pandemic (Table 48–1).[77,159]

Two other more notable foodborne human pathogenic species are *V. parahaemolyticus*, first identified in 1950 as the cause of gastroenteritis from consumption of contaminated dried sardines,[55] and *V. vulnificus*, an organism first isolated from a wound infection and described as a lactose-positive vibrio,[68] then given species designation in 1979.[48] *V. parahaemolyticus* has caused several large outbreaks of gastroenteritis from the consumption of contaminated seafood. This species can be considered as the leading cause of seafoodborne bacterial gastroenteritis, causing numerous individual cases besides the large recorded outbreaks.[126,137,168] *V. vulnificus* is considered to be one of the most invasive and rapidly lethal of human pathogens described.[127] Infections start with a gastrointestinal disorder and rapidly progress to a septicemic condition. *V. vulnificus* has received a tremendous amount of press coverage in the US in the past de-

Table 48–1 Cholera Pandemics

Pandemic	*Dates*	*Origin*	*Biotype*
I	1817–1823	Indian Subcontinent*	
II	1829–1851	Indian Subcontinent	
III	1852–1859	Indian Subcontinent	
IV	1863–1879	Indian Subcontinent	
V	1881–1896	Indian Subcontinent	Classical
VI	1899–1923/5	Indian Subcontinent	01 Classical
VII	1961–Present†	Indonesia	01 El Tor
VIII	1992–Present†	Indian Subcontinent	0139 serotype

* from Pollitzer[132]

† from Kaper et al.[77]

cade because of the high mortality caused in medically impaired individuals.

A majority of the *Vibrio* spp. have been identified more recently. Of the 34 species currently recognized, most were given species designation after 1950.[8] Molecular characterization of a number of these species has resulted in recent proposals to reclassify some either to a newly named genus or to a different one that already exists. Illnesses attributed to many of these species are rare and most likely have to do with host susceptibility. Most of these species cause sporadic, isolated illness and have not caused major outbreaks involving large numbers of individuals. The virulence mechanisms and the route of infection of several of these species have yet to be identified.

48.2 TAXONOMY AND CHARACTERISTICS OF *VIBRIO*

48.2.1 General Aspects

The family Vibrionaceae was first proposed in 1965 by Veron[7] to group organisms into genera that generally were oxidase-positive and motile by means of a polar flagellum. This grouping was not intended to imply ancestral relationship, but to differentiate the organisms from the Enterobacteriaceae and the recognized human pathogens in that group. Oxidase activity is the important character in differentiation of vibrios from the other common enteric pathogens. Most species in liquid media have a single, sheathed, polar flagellum. On solid media, some produce lateral, unsheathed flagella, which are essential for swarming on solid media. The family currently contains four genera: *Vibrio*, *Aeromonas*, *Plesiomonas*, and *Photobacterium*. *Vibrio* are Gram-negative, nonsporeforming, straight or curved rods and facultative anaerobes with both respiratory and fermentative metabolism.[8] All use D-glucose as a sole source of carbon and energy and most use ammonium salts as a sole nitrogen source. Bioluminescence has been observed in some species.

Vibrios are abundant in the aquatic environment. Several cause disease in humans as well as in marine vertebrates and invertebrates, such as fish, eels, and mollusks. Of the 34 currently recognized species, 12 have been associated with human infections. The disease spectra attributed to these 12 species are presented in Table 48–2.

Growth of all vibrios is stimulated by sodium ions, in concentrations of 5 to 700 mM. Most require 2% to 3% NaCl or a seawater base for optimum growth. Tolerance of various levels of NaCl in laboratory medium is used as a basis for species identification. Vibrios can be divided into two groups based on this requirement, those not requiring the addition of NaCl to laboratory media—*V. cholerae* and *V. mimicus*—and the remaining 32 halophilic species that require media supplemented with NaCl.

Many species tolerate moderately alkaline conditions, with growth at pH 9, and some at pH 10. They do not tolerate acidic conditions and some rapidly decline in numbers at pH 5 or less. Vibrios are also

Table 48–2 The Spectrum of Human Infections Caused by *Vibrio* Species

	Gastrointestinal	*Septicemic*	*Wound*	*Ear/Other Sites*
V. cholerae				
01 & 0139	X			
non-01/0139	X	X	X	
V. mimicus	X			X
V. parahaemolyticus	X			
V. vulnificus	X	X	X	
V. fluvialis	X			
V. furnissii	?			
V. hollisae	X			
V. alginolyticus	X		X	X
V. metschnikovii	?	X		
V. cincinnatiensis		X		
V. damsela			X	
V. carchariae				X

sensitive to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine), although the sensitivity differs between species. The sensitivity patterns are used in the differentiation of species.

A few exceptions in characteristics are notable for the food microbiologist. *V. metschnikovii* is the only species that is oxidase-negative. *V. furnissii* and *V. gazogenes* are the only aerogenic species, differing from the other species by producing gas during fermentation of glucose. It has also been reported that the O139 serogroup of *V. cholerae* is resistant to vibriostatic agent O/129.[77]

The biochemical properties of the clinically important species are published in a number of reference manuals.[8,44,84,109] Table 48–3 presents a list of the biochemical features for differentiation of the food-associated *Vibrio* pathogens and two other common seafood-associated organisms in the family Vibrionaceae (*Aeromonas hydrophila* and *Plesiomonas shigelloides*). *Vibrio* species can be grouped initially according to sucrose fermentation and the color reaction obtained on the sucrose-based thiosulfate-citrate-bile salts-sucrose agar (TCBS). *V. cholerae, V. fluvialis, V. furnissii, V. alginolyticus,* and *V. metschnikovii* are sucrose-positive. The sucrose-negative group includes *V. parahaemolyticus, V. mimicus, V. vulnificus,* and *V. hollisae,* although strain variability has been reported for some of these.

Table 48–3 Biochemical Characteristics of the Vibrionaciae Commonly Encountered in Seafood

	V. alginolyticus	*V. cholerae*	*V. fluvialis*	*V. furnissii*	*V. hollisae*	*V. metschnikovii*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*	*A. hydrophilia*	*P. shigelloides*
TCBS agar	Y	Y	Y	Y	NG	Y	G	G	G	Y	G
Oxidase	+	+	+	+	+	–	+	+	+	+	+
Arginine dihydrolase	–	–	+	+	–	+	–	–	–	+	+
Lysine decarboxylase	+	+	–	–	–	+	+	+	+	V	+
Growth in (w/v):											
0% NaCl	–	+	–	–	–	–	+	–	–	+	+
3% NaCl	+	+	+	+	+	+	+	+	+	+	+
6% NaCl	+	–	+	+	+	+	–	+	+	+	–
8% NaCl	+	–	V	+	–	V	–	+	–	–	–
10% NaCl	+	–	–	–	–	–	–	–	–	–	–
Growth at 42° C	+	+	V	–	nd	V	+	+	+	V	+
Acid from:											
Sucrose	+	+	+	+	–	+	–	–	–	V	–
D-Cellobiose	–	–	+	–	–	–	–	V	+	+	–
Lactose	–	–	–	–	–	–	–	–	+	V	–
Arabinose	–	–	+	+	+	–	–	+	–	V	–
D-Mannose	+	+	+	+	+	+	+	+	+	V	–
D-Mannitol	+	+	+	+	–	+	+	+	V	+	–
ONPG	–	+	+	+	–	+	+	–	+	+	–
Voges-Proskauer	+	V	–	–	–	+	–	–	–	+	–
Sensitivity to:											
10 μg 0/129	R	S	R	R	nd	S	S	R	S	R	S
150 μg 0/129	S	S	S	S	nd	S	S	S	S	R	S
Gelatinase	+	+	+	+	–	+	+	+	+	+	–
Urease	–	–	–	–	–	–	–	V	–	–	–

Y = yellow G = green NG = no or poor growth nd = not done V = variable among strains R = resistant S = susceptible

Source: Adapted from E.L. Elliot et al., *V. Cholerae, V. Parahaemolyticus, V. Vulnificus* and Other *Vibrio* Spp. in *Bacteriological Analytical Manual, 8th ed.,* pp. 9.01–9.27, A.O.A.C. International, Gaithersburg, Maryland, Food and Drug Administration.

Arginine dihydrolase and lysine decarboxylase activity are also useful to further differentiate the pathogenic species.

48.2.2 Serotyping of *V. cholerae*

Two important properties that identify epidemic *V. cholerae* are the production of cholera toxin (CT) and the somatic serogroup. Currently more than 150 serogroups have been identified antigenically and the majority of environmental strains encountered are in serogroups other than O1 and O139. The O1 serogroup has, until recently, been exclusively linked with epidemic and pandemic cholera. Until the emergence of the newly designated O139 serogroup, isolates identified as *V. cholerae* that would not agglutinate in O1 antiserum were reported as non-O1 *V. cholerae*. The determination of serogroups other than O1 and O139 is impractical; the more recent literature now refers to these as non-O1/O139 strains.

The O1 serogroup can be subdivided into the Ogawa, the Inaba, and the very rare Hikojima serotypes. The serotype distinction has been used for epidemiological purposes; however, recent reports indicate that serotype conversion can occur, so this distinction may prove of limited value. *V. cholerae* O1 also can be divided into two biotypes, classical and El Tor, based on hemolytic ability. Classical strains are nonhemolytic, while El Tor strains produce a beta-hemolysin detectable on sheep blood agar plates. Several other laboratory tests can be used to differentiate the O1 biotypes besides hemolytic ability, including a Voges-Proskauer reaction, inhibition by polymyxin B, agglutination of chicken erythrocytes, and a phage typing system.[8,44,84,109] The nonhemolytic classical strains are believed to be the cause of the first six pandemics and are endemic on the Indian subcontinent (Table 48–1). The El Tor biotype is responsible for the seventh pandemic, currently is the more prevalent of the biotypes, and is endemic in several areas of the world. Classical strains are isolated infrequently in Bangladesh since the advent of the seventh pandemic, seemingly being replaced by the El Tor biotype. The O139 serogroup is also hemolytic, resembling El Tor strains.

48.2.3 Serotyping of *V. parahaemolyticus*

The only other pathogenic species for which an extensive serotyping system has been developed is *V. parahaemolyticus*. There are currently 12 O (lipopolysaccharide) and 65 K (acidic polysaccharide) antigens recognized in Japan.[168] Many of the environmental and some clinical isolates are untypable by K antigen, but usually can be grouped by the O antigen. Pathogenic strains occur in each of the O groups, while a common O group may occur more frequently in patients in certain geographical regions.[2,82] While serotyping may be important for epidemiological purposes, commercial antisera are available only from Japanese manufacturers. In addition, they are too costly for most laboratories to purchase and, therefore, are not in use routinely.

48.3 CHARACTERISTICS OF DISEASES

48.3.1 *V. cholerae*

Cholera, recently reviewed in detail,[77] has an incubation period from several hours to 5 days after ingestion of food or water contaminated with toxigenic *V. cholerae*, dependent in part on the inoculum level. The infective dose of *V. cholerae* is believed to be about 1 million cells. Approximately 10^{11} cfu in buffered saline were necessary to induce diarrhea in volunteers;[101] however, only 10^6 cfu ingested in a sodium bicarbonate solution to neutralize stomach acid induced diarrhea. Volunteer feeding studies using 10^6 cfu in food such as rice and fish resulted in a 100% attack rate. This suggests that the ingested food can buffer the gastric acid, enabling the organism to reach and colonize the intestinal tract. *V. cholerae* multiplies in the small intestine and produces cholera toxin. A known predisposition to severe infections is hypochlorhydria.

Typical cholera is characterized by the sudden onset of vomiting and painless diarrhea, with the characteristic "rice-water" stools caused by the presence of mucous. This is followed by suppressed renal function, thirst, leg and abdominal cramping, and collapse due to marked dehydration and resultant electrolyte imbalance. Vomiting is common in patients, occurring a few hours after the onset of diarrhea. Profuse secretory diarrhea is the main symptom with resulting life-threatening dehydration. The fluid loss is so dramatic that an infected person can die within hours. These severe cases, cholera gravis, are reported to afflict about 15% of those infected by the classical strain and only about 2% infected by the El Tor biotype during the epidemics. Mild cases and asymptomatic infections occur most often. Moderate cases requiring medical attention occur in about 15% of those infected with the classical strains and only about 5% of those with El Tor infections. An infected person sheds millions of these organisms in their feces; thus the disease can spread rapidly in areas with poor sanitation and poor hygienic practices. Person-to-person contact has not been substantiated as a cause of spread of the disease. Immediate treatment by rapid infusion of intravenous fluids is usually successful for the advanced cases. Oral rehydration is generally used for the milder cases and has been described as one of the most important therapeutic interventions developed in the 20th century. Antimicrobial therapy, usually with tetracycline, has also been effective in shortening the duration of infection and reducing the carrier state; however, widespread use of antimicrobials is strongly discouraged due to development of resistant strains of the organism. Cholera initiates an immune response in humans and evidence exists for substantial infection-derived immunity. Several vaccines have been developed and some show promise for prevention of future epidemics. An easily administered vaccine, delivered orally, is desired. Currently, live, attenuated bacteria, killed whole-cell preparations, whole cell toxoids, and a Salmonella-based carrier of *V. cholerae* antigens are being evaluated in clinical trials.

There is an increasing number of reports during the past few decades that non-O1/O139 *V. cholerae* strains also cause human diarrheal illness and other clinical manifestations.[119,120] Gastroenteritis is the most common of the illnesses reported; however, these strains have also been isolated from wound and ear infections. Raw oyster consumption is correlated highly with the gastrointestinal infections. The great majority of non-O1/O139 strains do not produce CT and they are not associated with epidemics. Two foodborne outbreaks of gastroenteritis have been reported as caused by non-O1/O139 *V. cholerae*,[120] and there are numerous individual reports of illness implicating consumption of raw shellfish.[137] Gastroenteritis cases occur predominantly during the summer and fall months, accompanying the increased presence of these organisms in the environment. Generally, non-O1/O139 gastroenteritis is reported to be mild or moderate in severity; however, occasionally a severe cholera-like disease has been reported. Symptoms include diarrhea, abdominal cramps, fever, and less frequently, nausea and vomiting. There is no distinguishing symptom that would differentiate these infections from other gastrointestinal infections. Severe dehydration has been reported in a few cases. Duration of illness can be from 1 to 10 days and hospitalization and rehydration have been required for severe cases.

Non-O1/O139 strains have also been isolated from cases of septicemia.[123,138] The source of these infections was not identified in several of these cases. A few patients reported a recent history of seafood consumption or an association with seawater. The fatality rate reported for these cases is over 60%. Epidemiological evidence from some cases suggests that septicemia could be acquired by invasion through the intestinal tract, similar to invasion by *V. vulnificus* (discussed below). Several of these patients had underlying medical conditions that probably made them more susceptible to an invasive infection by rare strains of *V. cholerae*. Strains isolated from septicemic cases are reported to be heavily encapsulated.

48.3.2 *V. parahaemolyticus*

Gastroenteritis is also caused by *V. parahaemolyticus*. First identified in Japan,[55] this species has been the principal cause of foodborne gastroenteritis in that country for many years. Gastroenteritis caused by this species has been linked exclusively to consumption of contaminated seafood.[168] The illness usually begins acutely with explosive diarrhea and cramping abdominal pain, but subsides spontaneously within 48 to 72 hours. The incubation period ranges from 8 to 72 hours, with a median of about 18 hours. Other symptoms, reported in descending frequency, include nausea, vomiting, headache, low-grade fever, and chills. The organism causes damage to the gut mucosa and colonic ulceration; therefore, fecal leukocytes and the organism are found in stools. The mortality rate is quite low, death usually occurring only in the case of elderly or debilitated patients. A severe, dysenteric form of infection and bloody diarrhea occur occasionally. Severe cases of gastroenteritis may require hospitalization, although most are treatable with supportive therapy such as rehydration. Treatment with tetracycline has proven beneficial in cases of prolonged infection. *V. parahaemolyticus* rarely, if ever, invades the bloodstream. Volunteer feeding studies in Japan suggest that ingestion of 2×10^5 to 3×10^7 cells of the thermostable direct hemolysin-producing (TDH) strains are necessary to cause disease.[143]

48.3.3 *V. vulnificus*

V. vulnificus is an invasive species, and epidemiological evidence has clearly demonstrated an association between primary septicemia and the consumption of raw oysters.[127] This bacterium can induce a high fever, usually within 24 hours of ingestion, in susceptible hosts and results in a mortality rate exceeding 50%. The incubation period ranges from 7 h to several days. The most common symptoms include fever, chills, nausea, and hypotension. Symptoms typical of gastroenteritis including abdominal pain, vomiting, and diarrhea are reported more infrequently. The organism commonly becomes localized in the lower extremities with resultant necrosis in advanced cases. Untreated, death can occur within 3 to 5 days. To a great extent, survival depends on prompt antibiotic therapy, tetracycline being the most effective. For many of these infections, prolonged hospitalization was required for those surviving the disease. Surgical debridement and limb amputation were often necessary. Epidemiological evidence has identified individuals with an underlying, chronic disease to be at greatest risk from infection. Common medical histories include liver- or blood-related disorders such as alcoholic cirrhosis and hemochromatosis, which result in elevated serum iron levels. Other risk factors identified include gastric disease, renal disease, diabetes, and use of immunosuppressive agents. One specific susceptible group identified includes males over the age of 40 who abuse alcohol and consume raw oysters. The infective dose of this pathogen for the at-risk individual is unknown. Infections differ from those due to organisms causing true gastroenteritis in that large outbreaks have not been reported. An individual case usually occurs within a group of people or a family that may have consumed a particular lot of shellfish.

Wound infections caused by *V. vulnificus* range from mild to severe, some with rapidly progressive cellulitis and myositis.[127] These infections have occurred as a result of exposure to seawater. Antibiotic treatment of these wound infections with tetracycline has been successful. Again, surgical debridement often is necessary.

48.3.4 Other *Vibrio* spp.

Within the past 15 years, several newly designated species of *Vibrio* have been recognized as human pathogens, including *V. mimicus.*[31] This organism was formerly classified as an atypical, sucrose-negative variant of *V. cholerae.* Gastrointestinal isolates have been recovered from diverse geographical locations.[72] Raw oyster consumption was the most common suspect vehicle of transmission. Onset of symptoms was usually within 24 hours but ranged from 3 to 73 hours. Duration of some cases was as long as 10 days. The symptoms reported were diarrhea, nausea, vomiting, and abdominal cramps. Several cases of ear infection caused by this species have also been reported following exposure to seawater.

V. fluvialis[98] was described in 1981 and has been isolated occasionally from diarrheic stools. Most of the patients suffering diarrhea caused by this species had food histories that included raw oysters, although shrimp and cooked fish also have been implicated. Symptoms were similar to those of other intestinal infections by vibrios except for the frequent occurrence of bloody stools, suggesting possible invasiveness. *V. furnissii*, an aerogenic species closely related to *V. fluvialis*, was given species designation in 1983.[21] *V. furnissii* has been implicated in a few outbreaks of food-related gastroenteritis;[72] however, there is little clinical information concerning the role this species plays in diarrheal illness.

V. hollisae, first described in 1982,[63] has been isolated occasionally from diarrheic stools. The infection in most of these cases has, again, been associated with the consumption of raw molluscan shellfish.[1] Bacteremia has been reported in two instances although the source of these infections could not be identified. Most patients also had underlying medical conditions that probably increased susceptibility to infection. This species grows poorly or not at all on the selective agar TCBS, so in some clinical investigations this species may be inadvertently missed during specimen examination.

V. alginolyticus[139] is one of the most commonly isolated species from seafood and the marine environment, often at high levels. On rare occasions, it has been implicated in gastrointestinal illness[73] but is more commonly associated with wound and ear infections.[72] It has also been isolated from a respiratory infection. Virulence properties have not been defined and it is another vibrio whose role in seafoodborne illness is still unclear.

First described in 1888 and further characterized by Lee et al.,[99] *V. metschnikovii* has been isolated from seafood, river water, and sewage and from human stools, blood, urine, gall bladder, and a leg wound. This species' role as an agent of gastroenteritis has not been established. No evidence exists of the organism causing enteritis. Bacteremia has been reported occasionally but with limited case histories and the rare occurrence of severe illness,[61] it is difficult to determine whether the diseases caused by this species were food-related.

Because there are only two reported cases of infection by *V. cincinnatiensis*,[20] it is not clear whether this is a food-related pathogen. Both patients reported no prior contact with seafood or seawater. One case was of bacteremia and meningitis and the other of enteritis. Both patients were elderly and immunocompromised. The source of the organism was never determined.

Most of the human infections mentioned above are quite rare; however, these species are common in the aquatic environment. Clinicians and food microbiologists should be aware of these organisms and the possible illnesses they can cause. From a food standpoint, these organisms are not generally considered during routine analysis. Continued studies may show that these species contribute to gastrointestinal illnesses and identify potential virulence mechanisms.

48.4 MECHANISMS OF PATHOGENICITY

48.4.1 *V. cholerae*

CT, or choleragen, is perhaps the most intensively studied of any of the toxins. The ability of *V. cholerae* strains to produce CT is the major determinant of virulence. Isolates producing CT are considered fully virulent, are capable of causing epidemic cholera and should be reported as such. A toxin was first proposed in the late 1800s by Koch, but it was not until 1959 that a toxin was demonstrated by two groups working independently.[32,43] Ten years later Finkelstein and LoSpalluto[51] purified the toxin. This enabled further investigations of structure, receptor binding, and mode of action.

CT is a heat-labile protein toxin, composed of a single A and five B subunits, with an approximate molecular weight of 85,000 daltons.[77] The initial action of CT is binding of the B subunit to the receptor, ganglioside G_{m1}, on the cell membrane of epithelial cells. This binding is enhanced by the enzyme neuraminidase produced by *V. cholerae.* The A subunit, after proteolytic cleavage to two further subunits, stimulates adenylate cyclase, resulting in increased cellular levels of cyclic adenosine monophosphate (cAMP). Increased cAMP concentration leads to increased Cl^- secretion by intestinal crypt cells and decreased NaCl absorption by villus cells, resulting in electrolyte movement into the lumen. The resultant osmotic gradient causes water flow to the lumen. The massive water flow overwhelms the absorptive capacity of the intestine, resulting in diarrhea. Further mechanisms also may be involved in the secretory effects of CT. Prostaglandins and the enteric nervous system both respond to CT and may add to the secretion that occurs with this disease.

In addition to CT, *V. cholerae* O1 strains produce other toxins.[77] Zonal occludens toxin (ZOT) increases permeability of the small intestinal mucosa by decreasing tissue resistance of the intercellular tight junction, zonal occludens. It is hypothesized that ZOT causes diarrhea as a result of leakage of water and electrolytes into the lumen by hydrostatic pressure due to the increase in permeability. Sequences of the *zot* gene have subsequently been found in non-O1 strains. Accessory cholera toxin (ACE) causes fluid accumulation in ligated rabbit ileal loops and is suspected of forming an ion channel after its insertion into the epithelial cell membrane. The genes encoding these two toxins, *zot* and *ace*, are located immediately upstream of the *ctx* genes encoding CT in what has been termed a "virulence cassette." The hemolysin that differentiates the El Tor from classical strains is cytolytic to a variety of erythrocytes and mammalian cells in culture, is lethal to mice, causes fluid accumulation in ligated rabbit ileal loops, and is also produced by non-O1 strains. This hemolysin/cytolysin may play an accessory role in diarrhea produced by these strains. Volunteer feeding studies have shown that *ctx* gene-deleted O1 strains can still elicit mild to moderate diarrhea in some individuals; thus, these other toxins may be responsible for the diarrheal cases from which CT-negative strains were recovered. Other reported toxins produced by O1 strains include a "new cholera toxin," a sodium channel inhibitor, and a Shiga-like toxin. Some non-O1/O139 strains produce a heat-stable toxin, referred to as Nag-ST, similar to that produced by the enterotoxigenic *Escherichia coli.* The role of these toxins in human pathogenesis is unknown at present although they may contribute in part to diarrhea caused by strains that do not produce CT.

V. cholerae must first colonize the small intestine to begin the disease process. The toxin coregulated pilus, TCP, is the most characterized colonization factor of this organism. These pili consist of long filaments that are laterally associated in bundles. Expression of the pilus is correlated with the expression of CT; hence the name. Another potential colonization factor, accessory colonization factor, has been described and may be involved in colonization via motility and/or chemotaxis, but its exact nature is still under study. The polar flagellum of *V. cholerae,* besides being involved in motility, may serve as an adhesin and an important virulence factor. In response to chemotaxins, motile *V.*

cholerae are drawn to the mucosal surface of the intestine. Nonmotile but fully toxigenic strains show a markedly diminished virulence in animal models. The production of at least four hemagglutinins by *V. cholerae* has been reported and these may be involved in cellular adherence in the gut. Other possible adherence factors that have been described include outer membrane proteins (OMPs), the lipopolysaccharide (LPS) of the O1 strains, a polysaccharide capsule of non-O1 strains, and other pili. A siderophore, vibriobactin, is also produced by *V. cholerae* but is not necessary for virulence.

There are several systems for regulation of virulence genes in *V. cholerae.* The most extensively characterized regulon is ToxR, which controls expression of CT, the TCP colonization factor, the accessory colonization factor, two OMPs, and three lipoproteins. The control of 17 distinct genes by ToxR has been reported and it is called the "master switch." Volunteers fed a mutated *toxR* gene *V. cholerae* strain did not show diarrheal symptoms.

The virulence mechanisms of the occasional non-O1/O139 strains of *V. cholerae* associated with human infections remain unidentified for the most part. Strain-to-strain variability in virulence occurs within this group of organisms. Most strains fed to volunteers failed to produce illness. Inoculum levels of clinical non-O1/O139 strains greater than 10^6 were necessary to induce diarrhea in volunteers,[119] seemingly a greater number than required for the toxigenic O1 strains. No toxins unique to these serogroups have been described, although some produced by O1 strains and other species have been reported; for example, the hemolysin/cytolysin of the El Tor biotype and the thermostable direct hemolysin gene, *tdh*, of *V. parahaemolyticus* (discussed below). Some strains produce a heat-stable enterotoxin, Nag-ST (mentioned above), and a cholera-like toxin. A few non-O1/O139 strains possess the genes encoding CT, ZOT, and ACE. Other possible virulence factors reported are a Shiga-like toxin, various cell-associated hemagglutinins, and a polysaccharide capsule that could facilitate production of septicemia in susceptible hosts. Some strains of O139 *V. cholerae* have also been reported to produce a polysaccharide capsule, unlike the O1 serogroup. The production of multiple virulence factors by non-O1/O139 *V. cholerae* appears to be necessary to elicit a diarrheal illness, although host susceptibility may play an important role. However, case reports indicate that these bacteria have also caused gastroenteritis in healthy individuals as well as people with preexisting diseases.

48.4.2 *V. parahaemolyticus*

Stool isolates of *V. parahaemolyticus* from patients suffering gastroenteritis produced a beta-hemolysin under specific laboratory conditions.[116] This hemolysin was discovered by researchers in Japan; its production is termed the Kanagawa phenomenon and the strain is often reported as Kanagawa positive or KP+. More than 95% of clinical strains produced this hemolysin, characterized as a TDH. In contrast, only 1% of environmental strains in early studies produced TDH. The production of this hemolysin is detected by plating on a special high salt agar, Wagatsuma agar, containing freshly collected and washed human or sheep erythrocytes.[44,161] Various animal erythrocytes, but not those of horses, are lysed by TDH. TDH is an extracellular protein of approximate molecular weight 44,000 daltons with an apparent subunit structure. The mechanism of action is not understood. This hemolysin is lethal to adult mice on injection, causes fluid accumulation in mouse and rabbit intestines, and has other cytolytic and cardiotoxic abilities.[116,161] Using a KP+ strain and its isogenic KP– mutant, it was demonstrated that only the KP+ parent strain induced fluid accumulation in the rabbit ileal loop assay. When the *tdh* gene was replaced in the isogenic mutant, restoration of secretory activity was observed.

TDH was encoded by one or more nonidentical copies of the *tdh* gene, *tdh*1 and *tdh*2.[124] The predicted protein products differed slightly in amino acid residues, but the proteins were indistinguishable immunologically. KP+ strains contained two gene copies, while KP– or the weakly hemolytic strains only contained one gene copy. The KP phenotype and more than 90% of TDH produced under laboratory conditions were attributable to expression of the *tdh*2 copy. Several other *tdh* gene sequences have since been determined. The regulation of expression of these genes by a regulator similar to the ToxR of *V. cholerae* has been reported recently.[124]

A second hemolysin called the thermostable direct related hemolysin (TRH) was produced by strains of *V. parahaemolyticus* isolated from patient stools from an outbreak in Maldives.[69] These strains did not produce TDH. Two *trh* genes have since been sequenced and TRH has been purified. This hemolysin has characteristics similar, but not identical, to those of TDH.[70] Several clinical isolates of *V. parahaemolyticus* possessed both *trh* and *tdh* genes, but some strains possessed only the *trh* or only the *tdh* gene. Most of the environmental isolates examined contained neither gene. Illnesses previously reported as being caused by KP– strains possibly could be attributed to the production of this hemolysin. Development of an immunological test for TRH production, and a gene probe or polymerase chain reaction (PCR) procedure to detect presence of the *trh* gene, will aid in the assessment of potential virulence of strains of *V. parahaemolyticus* encountered in clinical specimens or seafood.

Several adhesion factors have been proposed for *V. parahaemolyticus*; among them are the lateral flagella, pili, a cell-associated hemagglutinin, and an OMP. The production of a siderophore has also been demonstrated. Further investigation into colonization factors and other virulence mechanisms of *V. parahaemolyticus* is needed.

A correlation has been reported between urease production and the presence of the *tdh* gene in strains of *V. parahaemolyticus* in the Pacific Northwest of the US.[82] All the strains from clinical and environmental samples that possessed *tdh* were urease-producing, but not all urease-producing strains possessed *tdh*. The production of urease may enhance the virulence of some bacteria. Urease was responsible for acid resistance by *Yersinia enterocolitica*.[33] The enzyme may contribute to virulence by enhancing the passage of the organism through the stomach, resisting gastric acid. Although this increase in acid resistance has not been demonstrated for *V. parahaemolyticus*, urease production may protect the organism during gastric passage. A correlation between urease production and the presence of the *trh* and *trh/tdh* genes in strains of *V. parahaemolyticus* also was recently reported in Japan[129,156] and in Thailand;[155] however, these investigators observed a lesser correlation between the production of the enzyme and the presence of *tdh*. Nonetheless, a simple laboratory test for urease production may help to screen for the potentially pathogenic strains.

48.4.3 *V. vulnificus*

The mechanism(s) of development of primary septicemia attributed to *V. vulnificus* are not completely understood.[127] Both clinical and environmental strains produce an extracellular cytotoxin-hemolysin, the more virulent strains producing higher titers under laboratory conditions.[166,167] This cytolysin, of approximate molecular weight 56,000 daltons, is hemolytic to mammalian erythrocytes, cytotoxic to Chinese hamster ovary (CHO) cells, affects vascular permeability of Guinea pig skin, and is lethal to mice. Because all strains possess the cytotoxin-hemolysin gene and this is specific to *V. vulnificus*, a gene probe procedure has been used for detection and identification of this bacterium,[176,177] but not for virulence assessment.[44,64] Some strains of *V. vulnificus* are highly lethal to mice in several different bioassays. Others are considered avirulent, requiring a significantly greater number of cells to kill mice. Thus the cytotoxin-hemolysin may only play a small role in virulence. *V. vulnificus* produces a variety of extracellular products including protease, elastase, collagenase, lipase, phospholipase, mucinase, hyaluronidase, and fibrinolysin; however, these have not as yet been demonstrated to be involved in pathogenesis.[127]

The presence of a polysaccharide capsule appears to be essential for the infection process, most likely conferring resistance to phagocytosis. Encapsulated cells can be demonstrated on nonselective laboratory medium, appearing as opaque colonies, while acapsular cells yield translucent colonies.[148] On subculture, acapsular cells can be obtained from encapsulated cells but not the reverse. The encapsulated cells are significantly more virulent to mice than are the acapsular form, based on LD_{50} assessment; acapsular forms have been reported as avirulent. This difference is important in the laboratory when selecting isolates for additional study and virulence assessment. At least 10 capsular polysaccharide serotypes of *V. vulnificus* have been identified.[146] Clinical isolates associated with primary septicemia were represented in each of the 10 serotypes; however, only 45% of clinical isolates and only 8% of environmental isolates were typable. The epidemiological value of this serotyping scheme is yet to be determined. Also to be determined is the extent to which encapsulated and acapsular forms exist in the environment and whether certain environmental conditions select for either form. Several studies have reported that environmental isolates are as virulent to mice as clinical strains, and highly virulent strains are frequently encountered in environmental samples.[86,152,166]

Five serological varieties of LPS of *V. vulnificus* have been identified,[146] but the chemical composition of only one LPS has been reported. The relative virulence of strains in each of these serotypes has yet to be determined. A partially purified LPS extracted from a strain on intravenous injection into rats caused rapid onset of hypotension, followed by decreased heart rate and death within 30 to 60 minutes.[111] Hypotension, along with fever, tissue destruction, and hemorrhagia, are classical symptoms of Gram-negative endotoxic shock. All of these symptoms are manifested in the primary septicemia caused by *V. vulnificus.* An LPS-induced enzyme, nitric oxide synthase, responsible for the release of nitric oxide and resultant tissue destruction, has been suggested as the cause of the toxic shock syndrome observed following infection by this pathogen.

Iron plays an important role in the pathogenesis of *V. vulnificus.* Using an iron-overloaded mouse assay, LD_{50} values of strains were reduced significantly compared with those of the same strain tested in untreated mice.[152,178] This response to serum iron coincides with the epidemiological findings that severe infections occur predominantly in individuals with elevated serum iron levels. Normal human serum inhibited the growth of *V. vulnificus* because the organism could not compete for transferrin-bound iron;[147] however, when transferrin was saturated with iron, virulent strains were able to compete for the iron and multiply. Virulent strains were also able to grow when two other iron-binding proteins, lactoferrin and ferritin, were iron-saturated. Only the virulent, encapsulated strains were able to grow. Two siderophores are produced by *V. vulnificus,* uncommon for *Vibrio* species, but their role in pathogenesis is undetermined.[127] *V. vulnificus* is able to overcome the binding of free hemoglobin by the serum protein haptoglobin and acquire heme iron. An extracellular protease contributes to the efficient utilization of heme by this organism.[125]

48.4.4 Other *Vibrio* spp.

Of the other *Vibrio* species involved in human illnesses, little is known regarding their possible virulence mechanisms.[72] Other than those mentioned above, potential virulence factors for these less common pathogens include heat-stable and -labile enterotoxins (*V. fluvialis, V. furnissii, V. hollisae*), cytolysins (*V. fluvialis, V. metschnikovii, V. damsela*), cytotoxin (*V. fluvialis*), thermostable direct hemolysin (*V. hollisae, V. mimicus*), and the enzymes collagenase (*V. alginolyticus*), protease (*V. mimicus*), and mucinase (non-O1 *V. cholerae, V. mimicus, V. fluvialis*). A cholera-like toxin essentially identical to CT was reportedly produced by two diarrhea-related strains of *V. mimicus.*[151]

48.5 INCIDENCE IN THE ENVIRONMENT AND FOOD

Vibrios are naturally occurring environmental bacteria, present in virtually all coastal waters of tropical and temperate regions of the world. Of the bacterial species encountered in this environment, the majority are vibrios. Coastal areas with brackish waters and estuarine regions are niches for many species, including strains of toxigenic O1 *V. cholerae.* Epidemic cholera strains are endemic in several regions including Australia and the US Gulf Coast, and are occasionally involved in illnesses in those regions. *V. cholerae* O1 strains are occasionally encountered in the environment in nonendemic areas, but they are normally nontoxigenic and considered to be nonpathogenic.[77] Ecological studies of *V. cholerae,*[78] *V. parahaemolyticus,*[76] *V. vulnificus,*[127,175] and *V. mimicus*[25] identified the physical factors that enhanced their incidence and distribution in the environment.

Water salinity is an important physical parameter in the ecology of these organisms. Several of the pathogenic species are more prevalent and found in higher numbers in areas where water has a general salinity range of 2 to 25 parts per thousand (ppt). The lower salinity range of 2 to 5 ppt, in areas with significant fresh water inclusion, favors *V. cholerae, V. mimicus,* and *V. vulnificus.* These three species are less prevalent or rarely recovered in water of salinity 30 ppt or greater found in the open ocean. *V. vulnificus* levels decreased by 58% and 88%, respectively, in water of salinities of 30 and 35 ppt.[79] Water samples of lower salinity and those collected closer to the bottom had increased concentrations of *V. vulnificus.*[175] Many estuarine areas fluctuate in salinity due to the amount of rainfall, to fresh water inclusion from streams or rivers, and to tidal action, which will also influence the incidence of these species.

Pathogenic species of *Vibrio* are seldom isolated from water at a temperature below 10° C but are more frequently isolated from water between 15° C and 35° C. During colder months, recoveries of some of these pathogens can be made only from sediment, where they may be insulated from the lower temperatures. *V. vulnificus* was not isolated from environmental samples when the water temperature was below 8° C; however, this organism was isolated from oysters when the temperature was 7.6° C.[175] The seasonal occurrence of vibrios in temperate regions is due to the effect of temperature changes and is correlated with the frequency of reports of infections. In regions where the water temperature is warmer year-round, this seasonality is not as dramatic.

The environmental presence of pathogenic vibrios does not distinctly correlate with the presence of human sewage, although the presence of sewage may add nutrients to the water that would enhance survival of many of these species. It is unlikely that a monitoring program that uses indicators of fecal pollution, such as *Escherichia coli*, would be effective in an area that is not subject to sewage pollution in indicating the presence of vibrio pathogens and the potential vibrio hazard in seafood, such as mollusks. Endemic *V. cholerae* strains, however, are more prevalent in regions where epidemics have occurred and there is an inclusion of human waste into the aquatic environment; thus, a monitoring program may identify potentially hazardous seafood gathered from those areas.

A relationship exists between several *Vibrio* species and the phytoplankton and zooplankton in the water. Vibrios are chitinoclastic and their ability to digest chitin may play a role in their persistence in the sediment. *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* are able to colonize copepods and crustacea due to the presence of chitin. It is suggested that attachment to planktonic forms enhances survival during adverse environmental conditions. *V. cholerae* also colonizes other aquatic biota such as water hyacinths in Bangladesh, seemingly extending its persistence in freshwater environments.[77]

Due to the ability of vibrios to attach to material suspended in the water, the animals that frequent these environments can be expected to pick up these vibrios during feeding. No particular animal reservoir has been identified; however, pathogenic vibrios are commonly found on surfaces or in the intestinal contents of marine animals. Seafood harvested from coastal and inshore areas of many parts of the world has been reported frequently to contain pathogenic species. In several of these reports, however, it was not indicated whether pathogenic strains, such as the KP+ *V. parahaemolyticus,* were encountered. A number of these reports are incidence studies and the levels of pathogens found were not reported. Products included in these studies were several species of finfish, squid, crustacea (shrimp, crab, lobster), and mollusca (oysters, clams, cockles, mussels, conch). The incidence of *V. parahaemolyticus* during processing steps of two oyster shucking operations in the State of Washington has been studied.[83] Levels of *V. parahaemolyticus* were traced from the freshly harvested shellstock, shucked oysters, washed and packed oysters, through the retail shucked product in jars. During a warm month of the year, 64% (91) of 142 total inplant samples collected contained *V. parahaemolyticus* with 20% of the 91 samples containing KP+ strains. Nearly one half of the final product jars contained KP+ *V. parahaemolyticus*; however, levels were less than 100 g^{-1}. In most samples, the total *V. parahaemolyticus* population exceeded that of the KP+ strains present; for example, shucked oysters prior to the wash step contained 2100 *V. parahaemolyticus* g^{-1}, 720 g^{-1} of these being KP+ strains. During the spring when the water temperature was lower, only 15% of 117 samples contained *V. parahaemolyticus*. KP+ strains were not recovered during this period.

There have not been any extensive surveys for the presence of pathogenic vibrios in food other than seafood. Due to the halophilic nature of these organisms, they would not be expected in other environments. In one of the first incidence studies for the presence of *V. metschnikovii* in seafood, the organism was isolated from a wide variety of products at the retail market level, including 12 common species of finfish, clams, oysters, mussels, scallops, shrimp, and a surimi-based product.[23] This organism appears to be quite prevalent in seafood; this may be of public health significance, although *V. metschnikovii* rarely causes human illness. The reservoir for *V. cincinnatiensis* has not been identified and, to date, no incidence studies in seafood have been reported. The presence of *V. metschnikovii* and the other species that are frequently encountered in seafood at the market level obviously indicates that the proper refrigerated storage of seafood is essential to prevent multiplication of potentially pathogenic vibrios.

Nonmarine food has been examined during cholera epidemics to determine modes of transmission, but routine incidence studies are not warranted. Foods investigated in epidemic areas include rice, millet gruel, vegetables, and fruit (see below). Imported vegetables from South America were examined for the presence of *V. cholerae* by several countries from 1992 to 1994 after the epidemic in that region intensified. These monitoring programs were initiated to prevent the possible spread of the strain outside Latin America. Several Latin American countries temporarily lost their European market for produce, creating a significant economic impact for the exporting country. Frozen seafood imports from South and Central America were also examined during this period. In the US, more than 600 imported seafood samples were examined. *V. cholerae* O1 was not isolated from any of the samples; however, non-O1 *V. cholerae* was isolated from 27, suggesting a risk that the epidemic strain could be conveyed to other regions.

48.6 EPIDEMIOLOGY

48.6.1 *V. cholerae*

Water plays a critical role in the transmission of cholera. Snow, in the mid 1800s, determined this role in England during the second pandemic.[132] The "Broadstreet Pump," a central supply of public drinking water in London, became infamous. The drinking water was contaminated with the epidemic strain by sewage discharge into the Thames River. The many investigations of cholera since Snow's discovery have consistently identified water as a source of the organism causing rapid spread of disease during epidemics.[80,115] In countries of recorded epidemics, the lack of potable water sources was the fundamental reason for spread of the disease. The contaminated water system of Trujillo, Peru, the drinking of unboiled water, and drinking from a household water-storage container in which hands had been introduced were all highly associated with the spread of cholera in that country.[160] Household water supplies, ie, cisterns used for collection and storage, often become contaminated by family members when washing their hands. During the 1991 South American epidemic, ice made from contaminated water was also incriminated in the transmission.[136] In Portugal, contaminated spring water that was bottled and distributed was responsible for a cholera outbreak of more than 100 cases.[15]

Food also plays a major role in the spread of cholera.[80,115,134] It is sometimes difficult to determine whether the food became contaminated by direct environmental contact or by an infected food handler, or whether the water used in food preparation was contaminated. There is evidence that water contaminated by sewage was used during preparation of food in outbreaks in the US, involving workers on an off-shore oil platform,[74] and also in Peru from prepared rice.[136] In South America, vegetables irrigated with untreated sewage were associated with the spread of that epidemic.[160] A variety of foods have been implicated in transmission of cholera (Table 48–4). Seafood implicated includes oysters, clams, mussels, cockles, crustacea, squid, and raw, salted, or dried fish. Other foods include cooked rice and legumes, millet gruel, raw vegetables, fruit, and frozen coconut milk. Ceviche, a dish made with raw marinated fish, was suspected of transmission in South America, but this was not substantiated. Fish used in the preparation of ceviche were harvested from coastal waters contaminated with the epidemic strain. In Ecuador, eating raw seafood and cooked crab was associated with the transmission of cholera, along with drinking unboiled water and a beverage from a street vendor.[173] Food obtained from street vendors was highly incriminated in the South American epidemic[164] and also in other countries during earlier epidemics.[102] In the US, the major domestic food vehicle for cholera in the past 25 years was crab meat.[172]

Illness caused by serogroups of *V. cholerae* other than O1 and O139 has most commonly been associated with the consumption of raw oysters in the US.[119] Outbreaks in other countries have been linked to the consumption of contaminated meat, vegetables, chopped eggs, potatoes, and gelatin.

48.6.2 Other *Vibrio* spp.

Illness due to *Vibrio* spp. other than O1 and O139 *V. cholerae* is consistently associated with a recent history of consumption of seafood. Many of the infections occur seasonally, in the warm months of the year, correlating with the increased occurrence of these species in the marine environment. Epidemiological evidence of seafood as a vector is lacking in some infections. Consumption of raw oysters is highly incriminated in epidemiological investigations of illness caused by most vibrios.[72,119,137] Indeed, the primary septicemia caused by *V. vulnificus* has been associated clearly with raw oyster consumption in more than 85% of cases.[127] The filter-feeding oyster can concentrate the bacteria present in the growing waters. High levels of the pathogenic vibrios have been reported seasonally in some of numerous incidence studies and this correlates with seasonal occurrence of most of the illnesses. Clams, cockles, and mussels also have been incriminated, but these mollusca are less often consumed raw than are oysters. In the US, consumption of molluscan shellfish accounts for the majority of gastroenteritis cases from seafood,[137] and *V. parahaemolyticus* appears to be the most common bacterial pathogen associated with shellfish consumption. Many of the documented cases involve a single incidence of illness and thus are not recorded as an outbreak, which requires two or more cases.[137] In a 2-year study of illnesses associated with raw oyster consumption in Florida, the reported disease agents in decreasing order of frequency were *V. parahaemolyticus*, non-O1 *V. cholerae*, *V. vulnificus*, *V. hollisae*, *V. mimicus*, *V. fluvialis*, and O1 *V. cholerae*.[89] Similar findings

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Table 48–4 Documented Outbreaks of Foodborne Cholera

Year	*Place*	*Number of Cases*	*Source*	*Reference*
1961	Philippines	330	Raw shrimp	75
1973	Italy	278	Raw seafood	5
1974	Portugal	2,467	Raw/undercooked cockles	14
1974	Portugal	136	Bottled water	15
1977	Louisiana (US)	11	Boiled/steamed shrimp	18
1978	Gilbert Islands	572	Raw clams, sardines	108
1981	Texas (US)	15	Cooked rice	74
1982	Singapore	37	Seafood	56
1987	Thailand	24	Uncooked pork	158
1988	Thailand	71	Uncooked beef	157
1991	Maryland (US)	3	Frozen coconut milk	165

were also reported for infections reported in one year in four Gulf Coast states.[100]

During the mid 1970s, oyster harvesting during summer months along the Gulf Coast region of the US was initiated due to the demand for the product. During this period, infections were recorded that eventually were attributed to *V. vulnificus*. During summer months, levels of *V. vulnificus* in oysters harvested from the Gulf of Mexico have been found to exceed 1 million g^{-1}.[162] These oysters are shipped to other areas of the US and large numbers are consumed raw. In most instances an individual consumes between 6 and 12 oysters or more; thus the number of *V. vulnificus* ingested at one time can be quite substantial. This suggests that several million of these organisms are consumed in raw oysters by some individuals without resultant illness. The attack rate, then, is seemingly quite low considering the estimated number of individuals that consume raw oysters. From 1981 through 1992, 72 *V. vulnificus* infections were reported in Florida, and this organism is the leading reported cause of death from foodborne illness in that state.[67] Eighteen cases of *V. vulnificus* septicemia have been reported for a 6-year period in Taiwan.[26] This bacterium infected two individuals with alcoholic cirrhosis in Thailand[174] and was reported as the cause of infection of four individuals in Korea in a 2-year period.[88]

In Japan, the high incidence of *V. parahaemolyticus* gastroenteritis results from the national custom of raw fish consumption. For a 10-year period (1963 to 1972), there were 300 to 500 outbreaks and 5,000 to 14,000 cases of illness due to *V. parahaemolyticus* yearly in that country.[126] In contrast, the large outbreaks of *V. parahaemolyticus* gastroenteritis in the US have been traced to cooked shrimp or crab that was contaminated after cooking at seafood processors. This may be due to a much lower frequency of raw fish consumption in the US than in Japan. In 1971, in Maryland, an estimated 320-case outbreak occurred from steamed crab,[117] and an outbreak involving an estimated 600 cases occurred in Louisiana from consumption of contaminated boiled shrimp.[6] Fresh shrimp, improperly stored, used in a salad served by a restaurant, caused acute gastrointestinal illness in 40 people in Panama.[93] Many of the US epidemiological reports regarding illness due to this organism have common factors involved; these include inadequate refrigeration (improper storage temperature, 75%), inadequate cooking (92%), and recontamination after cooking of the product (75%).[9]

It was estimated from a risk assessment study by the US Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention that the chance of contracting molluscan shellfishborne illness from all causes in the US was 1 for every 1,000 to 2,000 servings consumed raw; whereas the overall chance of seafoodborne illness was 1 in 250,000 servings.[114] Excluding raw shellfish consumption, accounting for approximately 85% of the seafoodborne illnesses, the chance of illness was estimated at 1 in 1 million servings. Thus, the elimination of raw shellfish from the diet would substantially reduce the number of seafoodborne infections and simply cooking all shellfish would drastically reduce the risk and the number of illnesses. Even so, the risk of illness from seafood consumption, including raw shellfish consumption, was estimated to be at least 10 times safer than for eating chicken in the US, estimated as 1 illness in 25,000 servings.

Increased travel and the transport of food products between countries has increased the potential for transmission of these organisms, as is shown by incidents such as the outbreak of cholera from imported coconut milk.[165] Cruise ships have been involved in several large outbreaks of *V. parahaemolyticus* gastroenteritis,[168] and food served on airlines has been implicated in cholera infections.[24,172] Travelers returning from countries with current epidemics or areas of endemic cholera have become infected from food consumed prior to departure or served on the airplane. In a few instances, travelers transported contaminated products in their baggage to another country. Cargo ships may have been responsible for transmitting the Latin American epidemic cholera strain to another country's coastal waters[35] via contaminated bilge water.[106]

Molecular subtyping techniques have very recently shown their value in identifying outbreak strains for epidemiological purposes. This is very evident in the results of subtyping epidemic strains of *V. cholerae* from Latin America and tracing the spread of the organism.[169] A standardized ribotyping scheme, based on restriction fragment length polymorphism (RFLP) patterns, has been proposed for *V. cholerae* O1 so that intralaboratory results are comparable.[133] PCR amplification of the CT gene was valuable also in detecting the epidemic strain in contaminated samples during epidemiological investigations.[50] Recently, the application of pulsed field gel electrophoresis (PFGE) has proven to be a very discriminating way to measure the RFLP of *V. cholerae*. Multilocus enzyme electrophoresis (MEE) is a useful epidemic marker system and also a way to measure the relatedness of *V. cholerae* strains.[169] These techniques identified two distinct strains of toxigenic *V. cholerae* O1 currently epidemic in Latin America.[46] The specifics of these techniques are discussed in Chapter 64. These techniques may soon be applied to other pathogenic *Vibrio* species to improve epidemiological investigations.

48.7 FACTORS AFFECTING SURVIVAL AND GROWTH

48.7.1 Temperature

Vibrios are mesophilic organisms; thus they thrive in the temperature range of 15° C to 40° C. No growth of *V. parahaemolyticus* was obtained at 10° C or 46° C.[4] The ability of some of these species to grow at 43° C has allowed for a temperature-selective approach to isolation[38] and is also used for identification.[44,84] Optimum growth is at 37° C for most of the pathogenic species. Several species have generation times of less than 20 minutes in some food[71] and 16 minutes in laboratory medium with 2.9% NaCl.[10] Although some strains may grow at 10° C or at slightly lower temperatures, they do not grow at refrigeration temperatures of 4° C or lower. Consequently, storage of seafood at recommended refrigeration temperatures (less than 4.4° C) immediately after harvest is essential to prevent an increase in numbers of vibrios. Generally, a slow decline in numbers is reported during extended storage. For example, for shellstock oysters in refrigerated storage, a steady decline of approximately a 4 $\log_{10}$ reduction was observed over 3 weeks for *V. parahaemolyticus* and over 2 weeks for *V. vulnificus*.[131] Postharvest storage at 10° C of oyster shellstock prevented multiplication of vibrios.[29] *V. parahaemolyticus* was recovered after 48 days from presterilized fish homogenate inoculated at approximately 10^7 g^{-1} and stored at 0.6° C, with a $\log_{10}$ reduction value of 3.8 for one strain.[105] It appears, then, that a refrigerated seafood may retain vibrios at a level that may cause illness for the approximate shelf-life of the product, at which time it would likely become unacceptable for consumption due to the natural bacterial and autolytic decomposition.

Freezing generally causes a reduction in levels of vibrios during storage, by 3 to 4 $\log_{10}$ units or greater. Recoveries after storage for 8 days at –20° C have been reported for *V. parahaemolyticus* in seafood,[168] after 3 months in rock lobster tails inoculated at 10^4 to 10^6 per sample and stored at –18° C,[94] and both *V. parahaemolyticus* and *V. vulnificus* were recovered from presterilized shrimp homogenate inoculated at approximately 10^8 g^{-1}, after 40 days' storage at –20° C and after 30 days at 4° C with about a 6 $\log_{10}$ reduction.[16] *V. vulnificus* was recovered after 10 weeks in naturally contaminated, frozen (–20° C), shucked oysters with an approximate 4 $\log_{10}$ reduction,[131] but only a 1.5 $\log_{10}$ reduction after 14 days at 3° C was observed in shellstock oysters allowed to naturally uptake *V. vulnificus* from inoculated seawater to about10^5 g^{-1}.[85] Significantly lower levels (4 $\log_{10}$ reduction) of *V. vulnificus* were recovered from vacuum-packaged oysters, inoculated at approximately 10^5 g^{-1}, after 70 days at –20° C than normally packaged oysters.[131] After inoculation at levels of 10^6 to 10^7 g^{-1} into shrimp, crab, and oyster homogenates, which were then stored at 7° C and –20° C for 21 days, the numbers of viable *V. cholerae* O1 were reduced between 10^3- and 10^5-fold.[135] Survival probably depends more on the freeze-thaw cycle and the protection offered by the food than the actual storage temperature. An increase in the NaCl concentration to a range of 3% to 12% (w/v) reportedly enhanced the tolerance of *V. parahaemolyticus* to cold temperature[30] and heat. The $D_{47°C}$ values in aqueous solutions of 0.5%, and 7.5% w/v NaCl were 0.8 and 6.5 minutes respectively.[12] Cells grown at increased temperature (21° C, 29° C, and 37° C) in broth containing increased concentrations of NaCl (0.5%, 3.0%, and 7.5%) showed greater heat resistance in a buffer containing the higher concentrations of NaCl.[12]

Other studies have shown that ambient-temperature storage of cooked shrimp, crab, octopus, and oyster shellstock promoted rapid growth of *V. parahaemolyticus* and that unchilled, oyster shellstock supported the growth of naturally occurring vibrios, including pathogenic species.[127,168] *V. cholerae* O1 strains rapidly increased by 3 to 6 $\log_{10}$ in 24 to 32 hours in cooked foods, such as rice, lentils, eggs, and raw vegetables even at 22° C.[92]

Vibrios are susceptible to heat, and mild heating of seafood reduces their numbers. Boiling rapidly destroys vibrios, and an internal temperature of 60° C for several minutes appears to be sufficient to eliminate the pathogenic strains, eg, causing a 10^4- to 10^5-fold reduction of *V. cholerae*.[17] Table 48–5 lists *D* values in minutes for three species in different suspending media. In rice and fish, a 10-min treatment at 60° C eliminated 10^4 to 10^5 *V. cholerae*[59] and a 30-second immersion of fruits and vegetables in boiling water killed the organism.[49] A pasteurization temperature of 57.2° C for 30 minutes reduced *V. cholerae* levels 4 to 5 $\log_{10}$ in shucked oysters.[17] *V. parahaemolyticus* was found to be highly susceptible to less than 2 minutes of microwave heating at 800 w.[62] Oysters naturally contaminated with *V. vulnificus* at a level of 6×10^4 g^{-1} were heated at 50° C for 10 min, resulting in a reduction in numbers of *V. vulnificus* to <0.3 most probable number (MPN) g^{-1}, the minimum detection level.[28]

48.7.2 Moisture and pH

Foods with a high moisture content allow the survival of *Vibrio* spp. The a_w value must be greater than 0.93. The minimum a_w, controlled by glycerol, for *V. parahaemolyticus* growth was reported as 0.937 and as 0.945 controlled by NaCl, incubated 20 days at 29° C.[10] In addition to the water content of a food, the surface humidity of a stored dried or semidried product is also an important factor.

Survival is better in foods of neutral or near neutral pH, such as seafood, than in foods with a pH of 5 or lower.[141] *V. cholerae* survived only 1 day at pH 5 but up to 3 weeks at pH 8 and 10 at room temperature in cooked eggplant inoculated at 2×10^4 g^{-1},[49] and no growth occurred in food with a pH lower than 5.5.[141] *V. cholerae* multiplied rapidly in peanut sauce (pH 6.0) but not in a tomato-based sauce (pH 5.0).[153] Acidic sauces used in preparation of certain seafood dishes[115] can restrict vibrio growth if in contact with the organism; however, there is unlikely to be much effect on the internal vibrios that would be present in filter-feeding shellfish. A pH of 4.8 adjusted with HCl was the minimum reported for growth of *V. parahaemolyticus* in tryptic soy broth with a total concentration of 3% (w/v) NaCl.[11] Proteinaceous food with a pH above 5.8 supported growth of *V. parahaemolyticus* even at 9° C, but not beans with a pH of 4.95.[122] Seafood cocktail sauce (pH 3.3 to 3.4) added to shrimp in a 2 to 1 ratio accelerated die-off of *V. parahaemolyticus*.[19] Seafoods are high in protein, contain the nutrients necessary for growth of vibrios, and thus must be considered highly perishable and handled as such.

48.7.3 Antimicrobials, Disinfectants, and Preservatives

Nine species of vibrio from more than 200 clinical and environmental sources tested for antimicrobial susceptibilities were reported to be resistant to sulphamethoxazole, trimethoprim, and the penicillins;[54] however, most were susceptible to tetracycline, chloramphenicol, the aminoglycosides, the quinolones, and the newer cephalosporins. Tetracycline is the antimicrobial of choice for the treatment of cholera,[77] *V. parahaemolyticus* infections,[168] and *V. vulnificus* septicemia and wound infections.[127]

Chlorine (100 ppm), a quaternary ammonium compound (50 ppm), and iodine (25 ppm) produced 6 $\log_{10}$ reductions of *V. cholerae* O1 strains suspended in phosphate buffered saline after exposure for 2 min.[107] The addition of 50 ppm chlorine to the washwater used in oyster processing failed to reduce the numbers of *V. cholerae* in the blower/washer within 5 to 10 minutes,[121] but chlorine and an iodophor tested separately at a concentration of 25 ppm reduced the numbers of *V. parahaemolyticus* by a factor of 10- to 100-fold during oyster processing.[57] The iodophor was reported to be more effective than chlorine at 0° C and did not affect the sensory or visual appearance of the oysters. The effect of these disinfectants will depend on the concentration in the washwater of protein and other compounds that will complex with the antibacterials and inactivate them.

Diacetyl at a concentration of 0.05% was reported to be an effective antimicrobial against *V. vulnificus*

Table 48–5 *D* Values (min) Reported for *Vibrio* Species*

	V. cholerae	V. parahaemolyticus	V. vulnificus
$D_{47^\circ C}$			2.40 (PBS-2% w/v NaCl)[28] 1.30 (oysters)[17]
$D_{49^\circ C}$	1.70 (1% w/v peptone)[145] 8.15 (crab)[145] 9.17 (shrimp)[66]	0.82 (clam)[34]	
$D_{50^\circ C}$			1.15 (PBS-2% w/v NaCl)[28] 0.66 (oysters)[17]
$D_{51^\circ C}$		0.66 (clam)[34]	
$D_{53^\circ C}$		0.40 (clam)[34]	
$D_{54^\circ C}$	1.04 (1% w/v peptone)[145] 5.02 (crab)[145] <0.43 (shrimp)[66]		
$D_{55^\circ C}$		0.29 (clam)[34]	
$D_{60^\circ C}$	0.63 (1% w/v peptone)[145] 2.65 (crab)[145]		
$D_{66^\circ C}$	1.60 (crab)[145] 1.22 (crayfish)[58]		
$D_{71^\circ C}$	0.30 (crab)[145] <0.30 (crayfish)[58]		

* seafood tested as homogenates.
Note: numbers in superscript indicate reference numbers

in oysters stored at 5° C;[154] however, lactic acid and butylated hydroxyanisole (BHA) in concentrations up to 0.05% were found to be ineffective under the same conditions.

48.7.4 Viable but Nonculturable Cells

Although toxigenic *V. cholerae* was reported to survive for up to 70 days in sea salt solution at 25° C,[113] *V. cholerae, V. vulnificus,* and several other Gram-negative bacteria have been reported to survive in a viable but nonculturable state (VBNC) (Chapter 15).[128] This phenomenon occurs when bacterial cells retain metabolic function but are not culturable on routinely employed nonselective bacteriologic media. This dormant state is induced by extended exposure to saline water and nutrient deprivation. Cells become ovoid and reduced in size (ie, the starvation response). The VBNC state has been induced in the laboratory by inoculation into solutions containing between 0.5% and 2.5% (w/v) NaCl and storage at 5° C. Detection of VBNC cells by epifluorescent microscopy is detailed in Chapter 15. Cells transform into a normal size and culturable state when environmental conditions such as temperature and presence of nutrients become favorable. In the VBNC state, cells retain virulence characteristics. VBNC cells of *V. cholerae* induced fluid accumulation on injection into ligated rabbit ileal loops, and VBNC cells of *V. vulnificus* were lethal to adult mice. The VBNC state is a plausible explanation for the persistence, undetected, of these pathogens in the environment during adverse conditions, such as the colder winter water temperatures in temperate regions. In addition, this dormant state may explain, at least in part, why endemic cholera strains cannot be recovered from the environment for periods of time, only to re-emerge and once again infect humans.

48.8 PRINCIPLES OF DETECTION IN FOOD

48.8.1 Enrichment Procedures

Media devised for the detection of pathogenic vibrios in food and water capitalize on the ability of these organisms to grow rapidly at an alkaline pH, resist the inhibitory effects of bile salts and sodium tellurite, and tolerate salt. The addition of 1% (w/v) NaCl to a medium lacking salt is required for vibrio growth, while a final concentration of 2% to 3% is normally used for the halophilic species.[44,84] The optimal NaCl concentration for *V. cholerae* was reported as 2% (w/v)[113] and 3% (w/v) for *V. parahaemolyticus*.[168] Nonselective enrichment broths have been used prior to the use of a selective agar to detect low levels of vibrios in food and to resuscitate stressed cells that can occur from certain processing steps, ie, drying, heating, freezing.

Vibrios tolerate moderately alkaline conditions; thus enrichment media have been formulated with the final pH between 7 and 9. The medium widely used for detection and enumeration in food or water is alkaline peptone water (APW) consisting of 1% (w/v) peptone and 1% NaCl with a pH adjustment to 8.4 to 8.6.[44,84] The sample, usually 25 g or 50 g, is added to 225 ml or 450 ml of broth. Incubation is at 35° C to 37° C for 16 to 18 hours. Other enrichment broths include gelatin-phosphate-salt and Monsur's tellurite-bile salts broths for *V. cholerae*[84,140] and salt-polymyxin broth and glucose-salt-Teepol (GSTB) broth for *V. parahaemolyticus.*[168] Teepol, a surfactant, is no longer commercially available, however. APW has been the choice of many laboratories, including the FDA,[44] for enrichment of *Vibrio.* APW was the recommended enrichment for *V. parahaemolyticus*[41] and was significantly more effective than salt-polymyxin broth for recovery of *V. parahaemolyticus* and *V. vulnificus* and cold-stressed cells from seafood.[60]

Vibrios tend to multiply more rapidly than other microflora that may be present and that interfere with isolation, and at 35° C to 37° C incubation times of 16 to 18 hours are recommended. It is recommended that the enrichment broth be incubated for only 6 to 8 hours for *V. cholerae* isolation.[44] For food that may have been frozen, dried, or subjected to heat, enrichment broths should be incubated after the 6 to 8 hours incubation for an additional overnight period to resuscitate stressed cells.[44] The optimum enrichment incubation time for *V. parahaemolyticus* in APW was reported as 8 hours at 35° C.[42] A duplicate enrichment of sample in APW incubated at 42° C also is recommended for isolation of *V. cholerae,* at least from shellfish samples, in addition to one portion of sample enriched at 35° C.[38,44] This elevated temperature method, which was adopted as first action by the Association of Official Analytical Chemists (AOAC) International,[37] takes advantage of the inhibition of growth at this temperature of many of the naturally occurring bacteria present.

48.8.2 Isolation Procedures

After incubation, the enrichment broth is then streaked to a selective or differential agar medium. The most commonly used is TCBS,[44,77,84,168] which is available commercially. This selective and differential medium was designed originally for detection of *V. cholerae,*[90] but has also been effective for isolating the other pathogenic strains. This medium will differentiate the sucrose-fermentative species such as *V. cholerae, V. alginolyticus, V. fluvialis,* and *V. furnissii* from the sucrose nonfermenting species, *V. parahaemolyticus, V. vulnificus,* and *V. mimicus.* TCBS is the recommended selective agar to be used in conjunction with another selective agar for vibrios.[41] Other selective agars that have been used successfully include Monsur's taurocholate-tellurite-gelatin agar, polymyxin-mannose-tellurite agar, and Vibrio agar for *V. cholerae;*[77,140] and bromthymol blue-sucrose-Teepol agar, salt-starch agar, and several others for *V. parahaemolyticus.*[168] We have used successfully both TCBS and cellobiose-polymyxin-colistin agar (CPC)[103] with a slight modification[44] for the isolation of *V. vulnificus* from seafood.[85] *V. vulnificus* is phenotypically similar to *V. parahaemolyticus* on TCBS agar, and the modified CPC agar enhances differentiation of these two species based on cellobiose fermentation; most *V. parahaemolyticus* strains will not grow on modified CPC. The combination of APW enrichment and CPC agar was reported as more efficient for recovery of *V. vulnificus* than other broth and agar combinations[149] and than by a direct plating of sample to a selective agar.[85]

48.8.3 Identification

Once isolates are obtained, determination of oxidase activity is of primary importance to distinguish presumptive vibrios. The laboratory procedures for biochemical identification are quite straightforward, although it is necessary to supplement the media used for biochemical identification with NaCl. The pathogenic species have been well characterized and tables of distinguishing characteristics are published in several manuals[8,44,84,109] and presented in Table 48–3. The traditional biochemical procedures can be time consuming, taking up to 10 days to complete isolation, identification, and virulence assay. For more rapid identification, several commercial diagnostic kits are available, such as the API 20E (bioMérieux Vitek, Inc., Hazelwood, MO; Marcy l'Etoile, France) used for enteric organisms, and have been used successfully. It is recommended that the suspension of the test isolate be prepared in a 2% NaCl solution for identification of the halophilic species.[44] The API 20E was determined to be a valid identification system for the more commonly occurring members of the Vibrionaceae.[130] A gas chromatographic procedure for *V. vulnificus* using a capillary column to analyze fatty acid profiles has been adopted as first action by the AOAC International for identification of this species.[95]

Samples are usually tested for the presence or absence of *V. cholerae.* If it is isolated, serological testing for either serotypes O1 or O139 is of primary importance, indicating the presence of a potential pathogen. Antisera for O1 and O139 are available commercially. Generally, no serological determination is made for the other somatic groups and they are reported as non-O1, non-O139 *V. cholerae.* Isolates of O1 or O139 serotypes should be tested for the production of cholera toxin, since nontoxigenic O1 strains are occasionally encountered. The mere presence in a food or water of a toxigenic O1/O139 *V. cholerae* constitutes a public health hazard. Cholera toxin production can be detected by several means, including effect of culture filtrates on cells such as Y-1 mouse adrenal tumor or CHO, production of intestinal fluid accumulation in infant mice, or immunological procedures such as the commercial Oxoid "VET-RPLA" reverse passive latex agglutination diagnostic kit.[44] Several monoclonal antibody-based coagglutination procedures for the detection of *V. cholerae* O1 in water and stool samples have become available commercially.[77] This approach to detection is currently being evaluated for foods. In addition, test kits to detect the O139 serogroup may soon be available.

An enumeration procedure is used for both *V. parahaemolyticus* and *V. vulnificus* in seafood. This is based on the presumption that these species can be encountered frequently and not all strains encountered in seafood are pathogenic; however, this may only apply to *V. parahaemolyticus*. The infective dose of KP+ *V. parahaemolyticus* is thought to be 10^5 to 10^7 cells,[143] and most routine samples do not contain high levels of the pathogenic strains. Recently, a significant genomic diversity among *V. vulnificus* strains isolated from one oyster was reported[22] and it is unclear at present whether all these heterogeneous strains are virulent. One oyster could contain diverse strains differing in virulence. Thus it would be difficult to assess the significance of a sample containing *V. vulnificus* based merely on its presence because not all strains have been determined to be virulent and the infective dose has not been determined. The number of *V. vulnificus* per gram of sample may be more informative to assess the potential risk if the seafood is destined to be consumed raw.

An MPN technique is commonly used to enumerate these two species using an enrichment broth for resuscitation of stressed cells.[44,84] A series of dilutions of the sample is inoculated into the broth tubes. The MPN procedure allows for a detection level of from 2 to 3 organisms g^{-1} of sample depending on the number of tubes used in the MPN series. A three-tube multiple dilution procedure is recommended.[44] APW, the recommended enrichment, is incubated 16 to 18 hours at 35° C to 37° C. Turbid tubes are streaked to TCBS and modified CPC. Isolates identified as *V. parahaemolyticus* should then be tested for TDH production on Wagatsuma agar because most strains encountered are considered nonpathogenic. Virulence of *V. vulnificus* isolates can be determined by animal bioassay using iron supplemented mice.[44] Results of analysis are reported as the most probable number of *V. vulnificus* or of KP+ *V. parahaemolyticus* g^{-1} of sample.

48.8.4 Other Procedures

Membrane filtration methods for enumeration of *V. parahaemolyticus* have been developed,[171] one using a hydrophobic gridded membrane, HGMF.[45] These offer an overnight incubation; thus, more rapid results than obtained by enrichment. The detection level would be 100 g^{-1} of sample. The HGMF procedure was more effective for enumeration than the membrane procedure of Watkins et al.[171] and GSTB enrichment procedure.[36] Large-volume water samples can be concentrated with a membrane filtration technique. Similarly, Moore swabs—cotton gauze suspended in water—have been used successfully to concentrate *V. cholerae* from water and sewage.[118] A direct plating procedure to enumerate *V. vulnificus* on a cellobiose-based agar was also reported.[112]

The advent of genetics-based identification methods for pathogenic vibrios offers the advantages in speed and specificity. Genetic probes are used for the detection of specific genes by colony hybridization.[64] These probes include sequences for the CT gene, *ctx*, of *V. cholerae* and the *tdh* gene of *V. parahaemolyticus* to determine potential virulence and the species-specific cytotoxin-hemolysin gene, *vvhA*, of *V. vulnificus* for identification. The probes are labeled with a radioisotope for detection of hybridization, which may limit their use in some laboratories. With the use of gene probes, *V. parahaemolyticus* could be differentiated from *V. vulnificus*, and both enumerated, using the HGMF procedure.[81] Recent advances in nonisotopic labels for DNA have led to a broader application of a hybridization procedure. An alkaline phosphatase-labeled probe for *V. vulnificus* could detect 20 cfu ml^{-1} in water and 100 g^{-1} in oysters using a direct plating technique on a nonselective agar,[176] a 4-day analysis. Gene probes for the *ctx* gene of *V. cholerae* can detect 10^4 cfu in samples.[77] Lee et al.[97] reported that 10^5 cfu g^{-1} of TDH+ *V. parahaemolyticus* was detectable in oysters within 3 days using a *tdh* gene probe. Currently, digoxigenin-labeled probes are being used for hybridizations. The sensitivity of some of these procedures needs to be enhanced to detect lower levels of these pathogens, particularly toxigenic *V. cholerae*.

PCR also offers rapid detection of pathogenic vibrios in food and water samples but, at present, does not allow enumeration. By amplification of DNA fragments by PCR, pathogens can be detected in approximately 24 hours, although specialized equipment is required. Procedures to detect toxigenic *V. cholerae*,[91] either KP+ *or TDH+ V. parahaemolyticus*,[96] and *V. vulnificus*[65] in food by PCR have been reported. The gene probe and PCR techniques are discussed in detail in Chapter 64.

48.9 CONTROL OF *VIBRIO* SPECIES

More than one control measure is necessary to prevent, or at least reduce, food- and waterborne vibrio infections. Control is basically accomplished through a procedure that eliminates or significantly reduces the chance that an individual will ingest an infectious dose. It is highly unlikely that vibrio illnesses will be eliminated completely because the major vehicle of transmission of most of these pathogens, seafood, is part of the diet of so many people of the world. Environmental reservoirs of pathogenic strains exist such that when environmental conditions favor their increased presence, seafood gathered from those areas will likely continue to cause illness. However, knowledge of the risk factors to the host, the mechanism of virulence of these pathogens and their ecology, and safe food handling can help to reduce the incidence of illness. One should assume that any seafood potentially presents a hazard for vibrio infections.

Seafood is highly perishable after harvesting; thus handling practices must prevent deterioration of the product and growth of *Vibrio* species. This would include temperature control at the initial point of harvest. The handling practices required are similar to those for other perishable food products and include refrigeration, heat treatment, separation of raw and processed products, and sanitation of contact surfaces and equipment. These practices apply to both a seafood-processing environment and the home. Commercial processors must adhere to strict sanitation and good manufacturing practices to ensure a safe product. Because seafood is exported by many countries, an outbreak can occur at a location far removed from the source. The adoption of, and adherence to, a hazard analysis critical control point program to ensure safety of fishery products by countries entering into trade agreements can eliminate some of the hazards.

Generally, the following handling practices apply to seafood as well as other perishable food during processing as well as in the home. Thoroughly cook seafood to destroy bacteria, then prevent cross-contamination of cooked seafood from raw product by separate handling of raw and cooked product. Use appropriate handwashing between handling of raw and cooked product. Keep raw and cooked product below 4.4° C before preparing and serving. Avoid eating raw or undercooked seafood.

Seafood is not always cooked thoroughly; for example, shellfish often are steamed for short periods of time until the shell opens. The cooking recommendation for live oysters in the shell is to boil 3 to 5 minutes after the shell opens or steam 4 to 9 minutes in a steamer already steaming.[52] Shucked oysters should be boiled for 3 minutes or until the edges curl, fried at least 3 minutes at 375° F (190.5° C), baked 10 minutes at 450° F (232° C), or broiled 3 minutes at least 3 inches (7.7 cm) from the heat source. After cooking, if seafood is not consumed immediately, it should be stored at temperatures below 4° C (40° F) or above 60° C (140° F) and protected from recontamination by vibrios. Large recorded outbreaks of gastroenteritis caused by *V. parahaemolyticus* were due to a cooked seafood that was contaminated by equipment that also was used to hold raw product.[9,168] Cooking of all seafood would require a change of certain cultural customs, as many recipes use raw product.

Individuals with liver or blood disorders and the other medical conditions mentioned in Section 48.4.3 should be strongly advised to eat only thoroughly cooked seafood. Elimination of raw oyster consumption would prevent many of the *V. vulnificus* infections as well as those caused by other species. Recently in the US, there has been an intensive effort to notify the individuals deemed at risk due to this pathogen. Information was sent to medical groups and distributed via consumer and trade publications. In two states, retail seafood outlets, including restaurants, are required to post a hazard statement regarding raw shellfish consumption.[114] This information deals mainly with the increased risk of infection by *V. vulnificus*; however, risk due to other species of *Vibrio* also is implied. In one state the retail posting reads, "Warning—raw oysters, raw clams, and raw mussels can cause serious illness in persons with liver, stomach, blood, or immune disorders."[114] It may be difficult to reach all these at-risk individuals and many may not be aware of a medical condition that predisposes them to infection. Recently, there has been a noticeable decline in the demand for raw oysters from the US Gulf region, and the educational program is seemingly somewhat responsible.

The environmental factors that result in the prevalence or increased levels of some of these vibrios in the water have been reported. A monitoring system based on water temperature and salinity might be used to identify the time of year in which the harvest of seafood from a particular area should be restricted.[162] An example is the US proposal to restrict the harvest of Gulf Coast oysters for raw consumption between April 1 and October 31 in an attempt to reduce *V. vulnificus* infections. Epidemiological data suggest that approximately 90% of these infections could be prevented by this harvest restriction.[53]

The practice of relaying molluscan shellfish to a clean water environment, used in many areas, and the process of commercial depuration using sterilized seawater has met with varying results for vibrio pathogens. Water temperature and salinity appear to affect the extent to which vibrios can be purged. Most of the studies indicate an extended time period is required to reduce vibrio levels. In clams, a 2 $\log_{10}$ decline in numbers of *V. vulnificus* was reported after 24 hours in ozonated, artificial seawater,[144] while in the Australian oyster, 9×10^5 g^{-1} *V. parahaemolyticus* could be reduced to 10^2 after one day and approximately 10^1 after two days in ultraviolet-treated seawater[150] and the incidence of *V. cholerae* in this species of oysters was reduced.[47] In one study of depuration in oysters, *V. vulnificus* shifted from the digestive tract to other tissues and was not purged from the animal.[163] At a water temperature exceeding 15° C, *V. vulnificus* was reported to increase in numbers in the oyster during "depuration." At least for some vibrios and some species of shellfish, these processes may offer little hope as control measures and also may not be economically feasible for shellfish harvested from certain regions.

The application of low dose gamma radiation (1.0 kGy) to seafood is another approach to reduce vibrios. Numbers of *V. vulnificus* were reduced by over 5 $\log_{10}$ in shellstock oysters with a low mortality rate to the animal;[87] a similar reduction occurred in the shucked product. During exposure to ^{60}Co, *V. parahaemolyticus* was the most radiation-sensitive of the foodborne pathogens studied with a D_{10} equaling 0.03–0.04 kGy[39] and 2.8 and 5.4 $\log_{10}$ reductions in crabmeat and fish homogenate with a dose rate of 5 krads min^{-1} (0.05 kGy) at 24° C.[104] Higher doses, 50 krad (0.5 kGy), reduced by 6 $\log_{10}$ the number of *V. cholerae* on frog legs.[142] The use of irradiation as a control measure may depend on economic feasibility and the acceptability by consumers of products treated by this method.

Control of cholera is a more formidable and costly task, but two measures can help to control the spread of disease or reduce the number of cases. Probably the most important measure is to protect potable or treated water, for example by utilization of a piping system or by ensuring limited access to the source of the water to reduce chance of contamination. Education of people in areas that are at risk to at least boil water prior to drinking or use for food preparation is also critical. Point-source chlorination of home water sources has been effective. In endemic areas, even modest improvements in food and water sanitation have resulted in fewer cases of infection. Disposal of human sewage so it cannot contaminate drinking water sources is the second important measure. In the Western Hemisphere, an estimated 80,000 metric tons of human feces is produced daily with only a small fraction being treated,[13] and cholera will probably become endemic in Latin America unless sewage treatment is implemented rapidly. Safer food-handling practices, safer food sources, and personal hygiene all can play a role in reducing transmission of cholera by both food vendors and by family household members. Adequate sanitation also requires uncontaminated water for cleaning and hygiene. Costs of community systems for potable water dispersal and for sewage disposal are most likely prohibitive in economically stressed countries. Development of an effective vaccine offers some hope in at least reducing the number of cases and the mortality due to this disease in regions where cholera is endemic. If O139 *V. cholerae* persists and spreads, a vaccine may have to be developed to include components of this serogroup; however, vaccines might be counterproductive if they decrease the motivation to improve sanitation.

48.10 CONCLUDING REMARKS

There are challenges ahead for public health officials to control vibrio infections. We have come a long way in identifying strains and virulence mechanisms of these organisms and recognizing the groups of people who are more susceptible to infection. The vibrio group contains species that are naturally occurring aquatic organisms. Humans are just an intermediate host, with some more susceptible to infections than others. For centuries, food derived from the sea has been a staple in diets of many populations. Vibrios will never be eliminated from their environment and as long as humans continue to consume seafood, illnesses will no doubt occur. The gastronomic delight, the oyster, is the cause for many of these illnesses, albeit unwittingly. Oysters fit the category of "you are what you eat." An understanding of the complete mechanisms of disease, the virulence factors involved, and their contribution to the disease process, obtaining a complete epidemiological picture of all vibrio illnesses, and conducting further research into vibrio ecology and possible new control measures are all ingredients needed in our attempt to reduce infections caused by vibrios and reduce the risk associated with seafood consumption.

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49

Yersinia species

Truls Nesbakken

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49.1 INTRODUCTION

The genus *Yersinia* of the family *Enterobacteriaceae* includes three well-established pathogens (*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*) and several non-pathogens.[122,166] *Y. pestis* was isolated by Alexandre Yersin in 1894.[273]

The most important *Yersinia* infection, plague, which is caused by *Y. pestis*, is one of the most ancient recognized human diseases. Although infections caused by *Y. pseudotuberculosis* and *Y. enterocolitica* have only been reported more recently, it is nevertheless likely that these infections also have occurred for many years. Disease due to *Y. pseudotuberculosis* (first described in 1884) has been recognized since the beginning of the 20th century, and *Y. enterocolitica* was first shown to be associated with human disease in 1939.[165]

The current interest in *Y. enterocolitica* started in 1958 following a number of epizootics among chinchillas and hares,[106,166] and after the establishment of a causal relationship with abscedizing lymphadenitis in man. The similarity between the human and animal isolates was established in 1963,[135] and in 1964 the species name *Y. enterocolitica* was proposed formally by Frederiksen.[73] During the past 30 years, the bacterium has been found with increasing frequency as a cause of human disease and from animals and inanimate sources.

Y. enterocolitica is an important cause of gastroenteritis in humans, especially in temperate countries.[166,267,268] The possible risk of reactive arthritis following infection with *Y. enterocolitica* has led to further attention being paid to this microbe.[35,166] In many instances, attempts to isolate *Y. enterocolitica* from foods implicated in cases of disease in humans have been unsuccessful. However, evidence from large outbreaks of yersiniosis in the United States (US), Canada, and Japan[58] and from epidemiological studies of sporadic cases[192,241] has shown that *Y. enterocolitica* is a foodborne pathogen, and that pork is implicated frequently as the source of infection.[106,147,169,192,241] An important property of the bacterium is its ability to multiply at temperatures near to 0° C and, therefore, in many chilled foods.

Due to the relative lack of information on *Y. pseudotuberculosis* as a foodborne pathogen,[217] this bacterium will be considered less extensively than *Y. enterocolitica*. *Yersinia pseudotuberculosis* mainly causes epizootic disease, especially in rodents, with necrotizing granulomatous disease of liver, spleen, and lymph nodes.[8,9] In humans it may cause acute abdominal disease, septicemia, arthritis, and erythema nodosum.[3,134]

49.2 TAXONOMY AND CHARACTERISTICS OF *YERSINIA* SPP.

The genus *Yersinia* was proposed in 1944 by Van Loghem[250] for bacteria that were related to the genus *Pasteurella* and that were pathogenic. Thal[244] drew attention to evidence relating *Yersinia* to the Enterobacteriaceae. A general numerical taxonomic study from 1958 placed *Yersinia* between *Klebsiella* and *Escherichia coli*.[225] The allocation of *Yersinia* to the Enterobacteria was further supported by Frederiksen in 1964.[73]

49.2.1 Differentiation of *Y. enterocolitica* from Other *Yersinia* spp.

A range of strains of *Yersinia* variants has been isolated from animals, water, and food.[106,120,149,166] Many of these bacteria have characteristics that deviate considerably from the typical pattern shown by *Y. enterocolitica* but that, nevertheless, show that the bacteria concerned can be classified as belonging to the genus *Yersinia*.[122,166] Such *Y. enterocolitica*-like bacteria have now been divided on a genetic basis into seven new species:[10,22,24,25,38,39,40,249,263] *Yersinia frederiksenii*, *Yersinia kristensenii*, *Yersinia intermedia*, *Yersinia aldovae*, *Yersinia rohdei*, *Yersinia mollaretii*, and *Yersinia bercovieri*.

The latter four species are not included as separate species in *Bergey's Manual of Systematic Bacteriology*.[23] For practical reasons, the common term "*Y. enterocolitica*-like bacteria" is applied to these seven species. The species name *Y. enterocolitica* is reserved for the most typical strains, and it is only within this species that pathogenic strains have been found. It is important therefore to be able to differentiate the seven new species from *Y. enterocolitica*. This can be achieved by applying nine biochemical tests as described in Table 49–1, the fermentation of rhamnose, melibiose, and sucrose being key characteristics. *Y. pseudotuberculosis* is also included in Table 49–1. A description of the characteristics of *Y. enterocolitica* is given in Table 49–2.

Y. enterocolitica is a Gram-negative, oxidase-negative, catalase-positive, nitrate reductase-positive, facultative, anaerobic rod (occasionally coccoid) 0.5 to 0.8 × 1-3 µm in size.[23] It does not form capsules or spores. It is nonmotile at 35° C to 37° C, but motile at 22° C to 25° C with relatively few peritrichous flagellae. Some human pathogenic strains of serovar O:3 are, however, nonmotile at both temperatures. In addition, the bacterium is urease-positive, H_2S-negative, ferments mannitol, and produces acid, but not gas, from glucose.

The genus *Yersinia* also includes two further species that will not be considered further here: *Y. pestis* and *Y. ruckeri*, which is a fish pathogen.

49.2.2 Biovars and Serovars of *Y. enterocolitica*

The bacteria that are classified currently as *Y. enterocolitica* do not constitute a homogeneous group. Within the species, there is a wide spectrum of biochemical variants.[22,122,166,233] Such variations form the basis for dividing *Y. enterocolitica* into biovars. A number of different biotyping schemes have been described;[38,122,233,257] however, in 1987 Wauters et al.[264] described a revised biotyping scheme (Table 49–3) that differentiates between pathogenic (biovars 1B, 2, 3, 4, 5) and nonpathogenic (only biovar 1A) variants. In this chapter, the term biovar has been used in accordance with the latest edition of *Bergey's Manual*.[23] The proposed biovar 6[264] is reclassified into two new species: *Y. bercovieri* and *Y. mollaretii* (Table 49–1).[263]

Table 49–1 Biochemical Characteristics of *Y. enterocolitica*, *Y. enterocolitica*-Like Bacteria, and *Y. pseudotuberculosis*

Characteristic	*Y. enterocolitica*	*Y. frederiksenii*	*Y. kristensenii*	*Y. intermedia*	*Y. aldovae*	*Y. rohdei* *	*Y. mollaretii*	*Y. bercovieri*	*Y. pseudo-tuberculosis*
Acid production from:									
D-cellobiose	+	+	+	+	–	+	+	+	–
D-melibiose	–	–	–	+	–	+	–	–	+
Raffinose	–	–	–	+	–	+	–	–	–
L-rhamnose	–	+	–	+	+	–	–	–	+
Sucrose	+	+	–	+	–	+	+	+	–
L-sorbose	+	+	+	+	–	–	+	–	–
Other tests:									
Breakdown of α-methyl-D-glucoside	–	–	–	+	–	–	–	–	–
Ornithine decarboxylase	+	+	+	+	+	(+)	+	+	–
Voges-Proskauer reaction	+	+	–	+	+	–	–	–	–

Incubation at 28° C.

+ = more than 90 % of strains showing a positive reaction.

– = less than 10 % of strains showing a positive reaction.

(+) = weakly positive.

* = reaction of *Y.rohdei* type strain ATCC 43380.

Source: Data from references 10, 21, 38, 122, 263.

Table 49–2 Characteristics of Y. enterocolitica

Property		*Reaction*
Acid production from:	Adonitol	–
	D-cellobiose	+
	Dulcitol	–
	D-glucose	+
	Malonate	–
	D-mannitol	+
	D-mannose	+
	D-melibiose	–
	Mucate	–
	Raffinose	–
	L-rhamnose	–
	Sucrose	+
	D-sorbitol	+
	D-trehalose	+
Other tests:	Arginine dihydrolase	–
	Motility, 22° C to 25° C	v*
	Motility, 35° C to 37° C	–
	Metabolism of citrate (Simmons')	–
	Phenylalanine deaminase	–
	Gas from glucose	–
	Breakdown of gelatin, 22° C	–
	Production of H_2S	–
	Production of indole	v
	Potassium cyanide (KCN)	–
	Lysine decarboxylase	–
	Methyl red	v
	Nitrate reduction	+
	Oxidase	–
	Ornithine decarboxylase	+
	Pigment	–
	Urease	+
	Voges-Proskauer reaction, 22° C to 25° C	+
	Voges-Proskauer reaction, 35° C to 37° C	–

The tests are performed at 28° C, unless stated otherwise.
+ = more than 90% of strains showing a positive reaction.
– = less than 10% of strains showing a positive reaction.
v = between 10% and 90% of strains showing a positive reaction.
* = some of the human pathogenic variants of serovar 0:3/biovar 4 are nonmotile or only slightly motile at 22° C to 25° C.

Source: Data from D.J. Brenner, Classification of *Yersinia Enterocolitica*, in *Yersinia Enterocolitica*, E.J. Bottone, ed., pp. 1–8, © 1981, CRC Press, Inc., and G. Kapperud and T. Bergan, Biochemical and Serological Characterization of *Yersinia Enterocolitica*, in *Methods in Microbiology*, Vol. 15, T. Bergan and J.R. Norris, eds., pp. 295–344, © 1984, Academic Press, Inc.

O- and H-antigens. *Y. enterocolitica* can be divided into serovars using O-antigens. So far, 76 different O-factors have been described in both *Y. enterocolitica* and *Y. enterocolitica*-like bacteria.[259,261] A few strains, however, cannot be typed by this system, and the number of described antigen factors is, therefore, likely to increase in the future. Fifty-four H-factors have been recognized [S. Aleksic, personal communication, 1995],[261] but H-antigen determination is carried out rarely and most studies are limited to the O-antigens. Most isolates of *Y. enterocolitica* possess H-antigens consisting of several subfactors. The H-antigens are monophasic and remain stable after repeated subculture and after storage as stab cultures.[7] The great variability in H-antigens allows differentiation of strains that cannot be distinguished by any of the other typing methods employed (Table 49–7). Even though the preparation and standardization of specific antisera are both time consuming and costly, H-antigen typing can be

Table 49–3 Revised Biotyping Scheme for *Y. enterocolitica*

	Biovar					
Parameter	*1 A**	*1 B*	*2*	*3*	*4*	*5*
Lipase (Tween-esterase)	+	+	–	–	–	–
Esculin/salicin 24h	+/–	–	–	–	–	–
Formation of indole	+	+	(+)	–	–	–
Acid from xylose	+	+	+	+	–	v
Acid from trehalose/NO_3 reduction	+	+	+	+	+	–
Pyrazinamidase	+	–	–	–	–	–
β-D-glucosidase	+	–	–	–	–	–
Voges-Proskauer reaction	+	+	+	+	+	(+)
Proline peptidase	v	–	–	–	–	–

Incubation at 28° C.
v = variable
+/– = delayed positive
(+) = weakly positive
* = nonpathogenic variants

Source: Reprinted with permission from G. Wauters, K. Kandolo, and M. Janssens, Revised Biogrouping Scheme of *Yersinia Enterocolitica, Contributions to Microbiology and Immunology*, Vol. 9, pp. 14–21, © 1987, S. Karger AG.

a valuable supplement to O-antigen typing and biochemical characterization of *Y. enterocolitica* in epidemiological investigations.

Correlation between Biovars, Serovars, Ecology, and Pathogenicity. The relationships between the biovars, the O-antigens, and the ecology of *Y. enterocolitica* and related species are presented in Table 49–4.[260]

Biovar 1A includes a large number of serovars that are found in the environment, in food, and occasionally in the digestive tract of animals and humans. They do not possess virulence properties and are of little or no clinical significance for man.

Biovars 1B, 2, 3, 4, and 5 are potential pathogens for man and animals. They exhibit pathogenic properties and, when freshly isolated, usually harbor the virulence plasmid. Strains of biovar 1B belong to a small number of pathogenic serovars, the most frequent being O:8, O:21, O:13a, and O:13b, whereas O:4, O:18, and O:20 occur less frequently. They have been isolated mainly in the US and were therefore called "American strains." Since the beginning of the 1990s, a few strains have been isolated outside North America in Europe, Japan, India, and Chile.[260]

Biovar 2 includes only two serovars, O:9 and O:5,27, which are pathogenic for man. O:9 is important in Belgium, Holland, and France, while O:5,27 is quite common in the US.[162,166,267]

Biovar 3 includes serovar O:1,2,3, which has been isolated mainly from chinchilla and rodents and rarely from man.[106,166] There are also a few serovars O:5,27 in this biovar.[260] In the beginning of the 1990s, a Voges-Proskauer-negative variant of biovar 3, belonging to serovar O:3, was isolated in Japan and East Asia and has become a prominent bio-serovar pathogenic for man in these countries.[260,261]

Biovar 4 contains only one serovar, O:3, which is the main pathogenic serovar for man, distributed worldwide. It is also isolated regularly from healthy pigs.[53,66,106,178,219,257,258]

Biovar 5 is of no importance for humans. It includes serovar O:2,3, which is found in hares and some other animals.[106,139,142,166,224,260]

Although some antigenic factors are linked to pathogenic strains, for example O:3, O:9, O:8, serotyping alone cannot be used to indicate pathogenicity because these antigenic factors also occur in nonpathogenic species or biovars. For instance, factor O:3 is common in *Y. frederiksenii* and may also be encountered in other species and in biovar 1A of *Y. enterocolitica*. Factor O:8 occurs in biovar 1A and in *Y. bercovieri*, and factor O:9 in *Y. frederiksenii*. Hence, antigenic typing of the strains should always be performed after appropriate biochemical characterization.

49.2.3 Serovars of *Y. pseudotuberculosis*

So far, 15 different O-groups have been described and 5 H-antigens defined in *Y. pseudotuberculosis*[28] [S. Aleksic, personal communication, 1995].

Table 49–4 Correlation between Biovars, O-antigens, and Ecology of *Y. enterocolitica* and Related Species

Species/Biovars	*O-antigens*	*Ecology*
Y. enterocolitica		
Biovar 1A	various antigens 0:5, 0:6, 0:7,8, 0:10, 0:13,7, 0:41,....	Ubiquitous
Biovar 1B	0:8, 0:4, 0:13, 0:21 (= Tacoma)	Man, "American strains"
Biovar 2	0:9, 0:5,27	Man (swine)
Biovar 3	0:1,2,3	Chinchilla, rodents
	0:5,27, 0:3 (Far-East)	Man (swine)
Biovar 4	0:3	Man (swine)
Biovar 5	0:2,3	Hares, rodents, goats
Y. intermedia	various antigens 0:4,33, 0:17, 0:40, 0:52,....	
Y. frederiksenii	various antigens 0:58, 0:16, 0:38, 0:3, 0:9,35,....	
Y. kristensenii	various antigens 0:11, 0:12, 0:16, 0:28, 0:61,....	Ubiquitous strains: soil, water, food, animals, occasionally man
Y. bercovieri	0:58, 0:8, 0:36,70	
Y. mollaretii	0:59, 0:62, 0:67	
Y. aldovae	0:17, 0:52	
Y. rohdei	0:38, 0:76	

Courtesy of G. Wauters.

49.3 FEATURES OF THE DISEASES CAUSED BY FOODBORNE *YERSINIA* SPP.

49.3.1 Clinical Symptoms of Infection with *Y. enterocolitica*

Gastroenteritis is by far the most common symptom of *Y. enterocolitica* infection (yersiniosis) in humans.[35,58,166,270] The clinical picture is usually one of a self-limiting diarrhea associated with mild fever and abdominal pain.[270] Nausea and vomiting occur, but less frequently. The portion of the gastrointestinal tract usually involved is the ileocecal region.[213] The colon also may be affected and the infection may simulate Crohn's disease, which has a different prognosis.[85,251] Occasionally the infection is limited to the right fossa iliaca in the form of terminal ileitis or mesenteric lymphadenitis, with symptoms that can be confused with those of acute appendicitis. In several studies of patients with the appendicitis-like syndrome, *Y. enterocolitica* has been found in up to 9% of patients.[3,17,115,157,185,194,212] Infections with serovars O:3 or O:9 are, in some patients, followed by reactive arthritis[2] [Chapter 55], which is most common in patients possessing the tissue type HLA-B27. Often, although not always, the patient has shown prior gastrointestinal symptoms. Other complications seen with *Y. enterocolitica* infection are reactive skin complaints, erythema nodosum being the most common. Many such patients have no history of prior gastrointestinal involvement. Septicemia due to *Y. enterocolitica* is seen almost exclusively in individuals with underlying disease,[35] while those with cirrhosis and disorders associated with excess iron are predisposed particularly to infection and increased mortality.

Clinical symptoms are influenced by the age of the patient.[35,270] Gastroenteritis dominates in children and young people, while various forms of reactive arthritis are most common in young adults, and most patients with skin manifestations are adult females.[270] In Scandinavia, there is a relatively high incidence of both reactive arthritis (10% to 30%)[266] and erythema nodosum (30%),[4] caused by serovars O:3 and O:9. These forms of the disease have been almost totally absent in the US, where O:8, historically, has been the most common cause of yersiniosis. This situation may change, however, as illness caused by serovar O:3 is on the increase in the US.[29]

Transient carriage and excretion of both pathogenic and nonpathogenic *Y. enterocolitica* may occur following exposure to the bacteria. High rates of asymptomatic carriage have been reported in connection with outbreaks,[238] although in surveys unrelated to outbreaks, *Y. enterocolitica* has been isolated from the stools of fewer than 1% of individuals.[185] In patients with *Y. enterocolitica* enteritis, the organism may be excreted in the stools for lengthy periods after symptoms have resolved. In a study of Norwegian patients, convalescent carriage of *Y. enterocolitica* O:3 was detected in the stools of 27 (47.4%) of 57 patients for a median period of 40 days (range 17 to 116 days).[191]

49.3.2 Clinical Symptoms of Infection with *Y. pseudotuberculosis*

The clinical manifestations of infection with *Y. pseudotuberculosis* may include enteritis, abdominal pain, lymphadenitis mesenterica, pseudoappendicitis, sepsis, reactive arthritis, and erythema nodosum.[3,8,9,134]

49.4 MECHANISMS OF PATHOGENICITY OF *Y. ENTEROCOLITICA*

49.4.1 Pathogenesis and Immunity

Human infection due to *Y. enterocolitica* is predominantly by the oral route. The minimal infectious dose required to cause disease is unknown. In one volunteer, ingestion of 3.5×10^9 organisms was sufficient to produce illness.[235] The incubation period is uncertain, but has been estimated as being between 2 and 11 days.[207,235]

Enteric infection leads to proliferation of *Y. enterocolitica* in the lumen of the bowel and in the lymphoid tissue of the intestine. Adherence to and penetration into the epithelial cells of the intestinal mucosa are essential factors in the pathogenesis of *Y. enterocolitica* infection.[32,57,161,199] Reactive arthritis and erythema nodosum appear to be delayed immunologic sequelae of the original intestinal infection.

In humans, infection with pathogenic strains of *Y. enterocolitica* stimulates development of specific antibodies. It is not known whether specific serum antibodies protect against reinfection with *Y. enterocolitica* organisms of the same or different serovars. The immunological response can be measured by a variety of techniques, including tube agglutination, indirect hemagglutination, enzyme-linked immunosorbent assay (ELISA), and solid-phase radioimmunoassay.[17] An indirect immunofluorescent-antibody assay has also been used;[48] however, ELISA is probably the most suitable and extensively applied method in the world today. Agglutinating antibodies appear soon after the onset of illness and persist for 2 to 6 months. Some serovars may be associated with illness without eliciting a detectable serological response.[3,247]

The serological diagnosis of *Y. enterocolitica* infection may be complicated by the existence of cross-reactions between *Y. enterocolitica*, most notably serovar O:9, and such organisms as *Y. pseudotuberculosis, Brucella, Vibrio, Salmonella, Proteus,* and *Escherichia coli.*[184,259,265] The interpretation may also be confounded by high prevalence of seropositive individuals in the healthy population. Of 813 Norwegian military recruits selected at random, 67 (8.2%) had antibodies against *Y. enterocolitica* O:3.[181] Patients with thyroiditis of an immunological etiology have an unexplained increased frequency of cross-reacting antibodies to *Y. enterocolitica.*[220] Detection of antibodies to plasmid-encoded proteins, *Yersinia* outer membrane proteins (Yops), by immunoblots, has been suggested as a highly specific means of demonstrating previous *Y. enterocolitica* infection.[104,231] Demonstration of specific circulating IgA to the Yops is indicative of recent or persistent infection and is correlated strongly with the presence of virulent *Y. enterocolitica* in the intestinal lymphatic tissue of patients with reactive arthritis.

49.4.2 *In Vitro* Indicators of Virulence

The Virulence Plasmid. Human pathogenic strains of *Y. enterocolitica* possess a special plasmid 40 to 50 megadaltons in size.[43,199] The presence of this plasmid is an essential, although not sufficient, prerequisite for the bacterium to be able to induce disease. Properties associated with the chromosome are also necessary for virulence. Corresponding plasmids are found in *Y. pseudotuberculosis* and *Y. pestis*, and there is thus a family of related virulence plasmids within the genus *Yersinia.*[199] The way in which the plasmid contributes to virulence has not been elucidated fully. The presence of this virulence plasmid has been associated with several properties, most of which are phenotypically expressed only at elevated growth temperatures of 35° C to 37° C.[199] The list of such plasmid-mediated and temperature-regulated properties includes: Ca^{++}-dependent growth,[83,198,200] production of V and W antigens,[198] spontaneous autoagglutination,[141,223] mannose-resistant hemagglutination,[127] serum resistance,[193,198] binding of Congo Red dye,[202] hydrophobicity,[140] mouse virulence,[179,193,209,218] and production of a number of proteins,[47,199,200] of which one is a true outer membrane protein (YadA, previously termed Yop1).[160] This true outer membrane protein forms a fibrillar matrix on the bacterial surface and mediates cellular attachment and entry.[32] It also confers resistance to the bactericidal effect of normal human serum and inhibition of the anti-invasive effect of interferon.

The mechanism of virulence is known to vary even between different serovars within *Y. enterocolitica*. Plasmid-containing strains of O:8, O:13a, O:13b, and O:21 are lethal to orally infected mice, whereas O:3, O:9, and O:5,27 are not.[179,193,209,218] These latter serovars, however, are capable of maintaining intestinal colonization for at least 1 week after orogastric challenge.[179]

The Chromosome. Elements encoded by the chromosome are also necessary for maximum virulence. The pathogenic yersiniae share at least two chromosomal loci, *inv* and *ail*, that play a role in their entry into eukaryotic cells.[161] The *inv* and *ail* gene products can be classified as adhesins because they mediate adherence to the eukaryotic surface. Unlike other previously characterized bacterial adhesins, they also mediate entry into a variety of mammalian cells.

49.4.3 Virulence Assays

Two of the above-mentioned characteristics, Ca^{++}-dependent growth and spontaneous autoagglutination, have been employed frequently as indicators of the pathogenic properties of the bacterium. These tests depend on the presence of the plasmid; however, the plasmid may be lost in the course of a prolonged isolation procedure. Other situations where loss of the plasmid may occur are cultivation on magnesium oxalate agar at 37° C[198] and extended storage at refrigeration temperatures.[26] A test for pyrazinamidase activity has been included in the new biotyping scheme of Wauters et al.[264] (Table 49–3). The test is simple, reproducible, and shows a striking correlation between potential pathogenicity and lack of pyrazinamidase activity, independent of the presence of the virulence plasmid. The reliability of this test has been documented following studies of more than 1,000 *Y. enterocolitica* isolates representing a broad range of serovars and different geographical origins.[117] Pyrazinamidase activity is commonly found in all environmental strains, which never harbor the virulence plasmid. There are a few exceptions in biovar 1A strains that lack pyrazinamidase activity. Furthermore, in *Y. kristensenii*, the reaction often is weak. Pathogenic strains, whether or not the plasmid is still present, show a negative reaction. Also the invasiveness in HeLa cells is encoded chromosomally.[218] Only pyrazinamidase-negative, pathogenic strains are invasive for HeLa cells,[218] although they do not multiply within the cells.[64]

49.4.4 Enterotoxin Production

Many strains of *Y. enterocolitica* and related species produce a heat-stable enterotoxin (YEST) when the bacteria are cultured at 20° C to 30° C.[119,173,195] Certain strains, especially within the species *Y. kristensenii*, are also able to produce YEST at 4° C and 37° C.[119,126,173] This property is regulated by chromosomal genes and is independent of the virulence plasmid.

The toxin of *Y. enterocolitica* resembles the heat-stable enterotoxin (ST) of *E. coli* in pH stability, activity in suckling mice and in the rabbit ileal loop,[37] and the stimulation of guanylate cyclase activity;[112] however, unlike the ST of *E. coli*, the YEST is found only in the methanol-soluble fraction.[252] The toxin is not inactivated by exposure to 121° C for 30 min or by storage at 4° C for at least 5 months.[37] It is also highly resistant to enzymatic degradation,[188] but sensitive to treatment with 2-mercaptoethanol.[252] Purification of YEST reveals a protein with a molecular weight of about 3,000, and a high degree of amino acid homology with *E. coli* ST.[240]

Although there is no evidence to support the involvement of YEST in the pathogenesis of *Y. enterocolitica* enteritis, the possibility still remains that enterotoxigenic strains may produce foodborne intoxication by means of preformed enterotoxins.[119,126] This assumption is based on the fact that YEST is able to resist gastric acidity as well as temperatures used in food processing and storage without losing activity.[37] The ability to produce YEST at room and refrigeration temperature may give the "environmental" yersiniae a new significance in food hygiene as potential agents of foodborne intoxication.

49.5 INCIDENCE IN THE ENVIRONMENT AND IN FOODS

49.5.1 Incidence of *Y. enterocolitica* in Animals

Food Animals. Healthy pigs often are carriers of strains of *Y. enterocolitica* that are pathogenic to humans, in particular strains of O:3/biovar 4 and O:9/biovar 2.[106,120,217] Healthy pigs have been found to be infected with *Y. enterocolitica* O:3 in frequencies up to 85%.[53,66,106,174,175,178,219,221,257,258] The organisms are present in the oral cavity, especially the tongue and tonsils, and in the intestine and feces. Workers in Japan reported that O:3 strains were isolated from 85% of oral swabs from 40 freshly slaughtered, healthy pigs and presented evidence that the organism colonized the pigs' tonsils.[221] In this study, 34 (24.3%) of 140 pigs were carriers of the organism in the cecum, with counts ranging from fewer than 300 to 110,000 *Y. enterocolitica* g^{-1} of cecal contents. Strains of O:3 have been found frequently on the surface of freshly slaughtered pig carcasses (in frequencies up to 63.3%).[13,175] This is probably the result of spread of the organism via feces and intestinal contents during slaughter and dressing operations. The association between yersiniosis in humans and the consumption of raw pork in Belgium[241] and the apparently rare incidence of the infection in Moslem countries,[212] where consumption of pork is restricted, point to pork as a source of infection with *Y. enterocolitica*.

Other pathogenic strains do not appear to be as closely associated with pigs and may have a different ecology. In western Canada, O:8 and O:5,27 strains have been found most commonly in humans, but only O:5,27 strains were found in the throats of slaughter-age pigs.[217] In the US, O:5,27 strains were isolated from the cecal contents and feces of 2 of 50 pigs at slaughter.[138] Serovar O:8/biovar 1B, until recently considered to be the most common human pathogenic strain of *Y. enterocolitica* in the US[190] and in western Canada,[248] has seldom been reported in pigs.

Serovar O:3 was isolated from the rectal contents in 2 (3.0%) of 66 lambs in New Zealand.[45]

Surveys in New Zealand have found deer to carry both O:5,27/biovar 2 and O:9/biovar 2 [S. Fenwick, personal communication, 1996]. Antibodies against serovar O:9 have been found in sera from cattle in Belgium.[265]

In Norway, Krogstad[139] demonstrated outbreaks of *Y. enterocolitica* infection in goat herds in which serovar O:2/biovar 5 was implicated. He also described a case in which an animal attendant was infected by the same serovar. Biovar 5 has been isolated also from goats in New Zealand.[142] Enteritis in sheep and goats resulting from infection of *Y. enterocolitica* O:2,3/biovar 5 is also seen in Australia.[224]

Other Animals. Pigs, dogs, cats, and rodents, such as rats, may also occasionally be fecal carriers of O:3 and O:9.[71,82,99,106] The relative intimate contact between man and pets suggests a potential reciprocal transmission, although such an epidemiological link has not been clearly confirmed.[181,192] However, cats may have some importance as vehicles of transmission of *Y. enterocolitica* O:3 in pig herds.[222] O:3 has been isolated from 1 (0.9%) of 117 crows in Japan, and O:9 from one (0.7%) of 156 Japanese serows.[131]

It has been suggested that rodents may be reservoirs of O:8 and O:21 strains in North America,[217] and evidence has been published that this is indeed the case in Japan.[99] Serovars O:8 and O:21 are related closely in many ways (ie, biochemical profile, H antigens, animal virulence). Serovar 21 ("O:Tacoma") has been isolated from wild rodent fleas in the western US.[203]

As is the case with human pathogenic variants, the animal pathogenic strains also belong to particular combinations of serovars and biovars.[106,166] Serovar O:1/biovar 3 was responsible for widespread outbreaks in chinchilla both in Europe and the US during 1958–1964.[106,166] During the same period, epizootics were observed among hares along the French-Belgium frontier caused by serovar O:2/biovar 5.[106,166]

49.5.2 Incidence of *Y. pseudotuberculosis* in Animals

Y. pseudotuberculosis mainly causes epizootic disease, especially in rodents, with necrotizing granulomatous lesions in liver, spleen, and lymph nodes, infections in animals often being fatal.[8,9] *Y. pseudotuberculosis* has been isolated from both wild and domestic mammals as well as birds.[8,9] Rodents kept as fur animals, laboratory animals, and zoo animals, and birds kept in zoos and bird shops and as farm animals or pets are infected frequently.[8] Yersiniosis in deer in New Zealand is caused by *Y. pseudotuberculosis*, the occurrence of the disease correlating well with periods of stress, especially the nutritional and climatic stresses associated with winter.[101]

49.5.3 *Y. enterocolitica* in Environmental Reservoirs and Water

Although a range of different serovars and biovars has been isolated from the environment (eg, soil and water),[118,120,121,166] most belong to biovar 1A, according to the revised biotyping scheme described by Wauters et al.[264] (Table 49–3). As regards the so-called *Y. enterocolitica*-like bacteria, which also are common in the same environments, no clinical significance is attributed to most of the strains within biovar 1A;[35,166] however, such environmental strains have occasionally caused disease in immunocompromised patients.[35,166]

Shallow wells in particular, and rivers and lakes, are susceptible to contamination by surface runoff from rain or snow melt. Such runoff may become fecally contaminated by wild or domestic animals or by leakage from septic tanks or open latrines in the surrounding areas. Water is a significant reservoir of *Y. enterocolitica*;[41,98,125,143,144,210] however, most isolates of *Y. enterocolitica* and *Y. enterocolitica*-like bacteria obtained from water are variants with no known pathogenic significance to man.

Evidence that water supplies can be a source of pathogenic *Y. enterocolitica* has been found in certain outbreaks associated with contamination from water sources, and in a few sporadic cases, as well as case-control studies (Section 49.6.1).

49.5.4 Incidence of *Y. enterocolitica* in Foods

Pork. In contrast to the frequent occurrence of the bacterium in pigs and on freshly slaughtered carcasses, pathogenic *Y. enterocolitica* have only exceptionally been found from pork products at the retail sale stage, with the exception of fresh tongues.[61,63,177,180,217,243,262,268]

This phenomenon might be explained by the lack of proper selective methodology for the isolation of pathogenic strains; however, Wauters et al.[262] described a method that allowed *Y. enterocolitica* O:3 to be recovered from as many as 12 (24%) of 50 ground pork samples. Genetic probes can be used in DNA colony hybridization to demonstrate virulent *Y. enterocolitica* strains. The results of one such investigation indicate that traditional culture methods lead to underestimation of the presence of virulent *Y. enterocolitica* in pork products. A significantly higher detection rate (60%) was achieved when two isolation procedures[187,262] (Section 49.8.2) were combined with colony hybridization than when the isolation procedures were employed alone (18%).[180] In this study, counts of virulent yersiniae in pork sausage meat varied from 50 to 2,500 g^{-1}, and in pork cuts from 50 to 300 g^{-1}. A total of 150 samples of raw pork products from different slaughterhouses in Norway were analyzed by both a traditional culturing method[187] and a nested polymerase chain reaction (PCR) method.[130] *Y. enterocolitica* O:3 was isolated from two (1.3%) of the samples using the traditional method. The PCR results indicated the presence of pathogenic *Y. enterocolitica* in 39 (26%) of the samples.[116]

Ostroff et al.[192] showed in a case-control study that people with *Y. enterocolitica* infection reported having eaten significantly more pork or sausages than their matched controls. This might be explained by recontamination of sausages after pasteurization, bacterial multiplication during storage, and insufficient heat treatment in the kitchen.

Beef and Lamb. With a few exceptions,[45,265] cattle and sheep are generally not considered to be carriers of human pathogenic *Y. enterocolitica*. Nevertheless, the possibility exists of cross-contamination to beef or lamb from pig carcasses and pork in slaughterhouses, cutting plants, meat processing establishments, and butchers' shops. Beef is sometimes consumed after little or no heat treatment, and some concern has therefore been expressed about *Y. enterocolitica* contamination in beef. According to the case-control study of Ostroff et al.,[192] people with yersiniosis were more likely than controls to report a preference for eating meat prepared raw or rare.

In an extensive survey in Germany,[150] four strains of *Y. enterocolitica* and 6 of *Y. enterocolitica*-like bacteria were isolated from 37 samples of beef. Two of the beef isolates belonged to biovars that can cause typical human illness, but their virulence was not tested.[150] Environmental biovars of *Y. enterocolitica* were isolated from 15 of 61 samples of beef in Japan.[109] In the US, environmental biovars of *Y. enterocolitica* were recovered from 10 of 98 samples of vacuum-packaged beef and from 2 of 18 samples of vacuum-packaged lamb.[95] From a total of 100 samples of red meat, 50 each of beef and lamb from retail stores in Brisbane, Australia, only strains not harboring the virulence plasmid were obtained. Among the beef isolates, the strains belonged to *Y. enterocolitica* biovar 1A ($n = 21$), and *Y. frederiksenii* ($n = 4$), while the strains from lamb were categorized as *Y. enterocolitica* biovar 1A ($n = 12$), *Y. intermedia* ($n = 3$), and *Y. frederiksenii* ($n = 1$).[107] Ternström and Molin[243] found two nonvirulent *Y. enterocolitica* strains in 45 samples of beef.

Poultry. Stengel[228] isolated *Y. enterocolitica* serovars O:3 ($n = 3$), O:9 ($n = 3$), and nonvirulent *Y. enterocolitica* ($n = 13$) from 130 samples of poultry. This is probably the first time these virulent serovars have been isolated from poultry, and there was no obvious opportunity for cross-contamination from pork. Nevertheless, according to other investigations, poultry has not been proven to be a carrier of pathogenic *Y. enterocolitica*. Leistner et al.[150] isolated 35 strains of *Y. enterocolitica* and 56 related bacteria from 121 samples of chicken meat in Germany. Inoue and Nagao[110] recovered 16 strains of *Y. enterocolitica*-related bacteria from 40 samples of chicken in Japan. Nonvirulent *Y. enterocolitica* was also recovered from 3 of 75 turkey meat samples in the US[90] and from 2 poultry samples in Quebec, Canada.[49] Norberg[186] isolated 24 *Y. enterocolitica* strains from 82 samples of frozen chicken. The author regarded the isolates as environmental strains even though 1 of them agglutinated as serovar O:8. Unfortunately, no additional biotyping or virulence assay was done. Nesbakken et al.[181] failed to recover a single *Yersinia* isolate from a total of 360 poultry from eight flocks that were sampled by cloacal swabs in one poultry processing plant in Norway. In the US, 60 ready-to-cook broiler carcasses obtained from several local supermarkets were tested for the presence of *Yersinia enterocolitica*. None of the 16 *Y. enterocolitica* isolates was found to be presumptively virulent.[59]

Milk and Dairy Products. Worldwide studies indicate that *Y. enterocolitica* is fairly common in raw milk,[149] and several outbreaks of yersiniosis due to serovars O:3, O:6,30, O:8, O:10K, and O:13,18 have been associated with the consumption of milk or milk products.[12,30,52,88,170,239] *Y. enterocolitica* was isolated from ice cream[168] and pasteurized milk[214,274] as early as 1970.

Reported levels of raw milk contamination with *Y. enterocolitica* vary: Japan, 4%;[81] Denmark, 10%;[54] Australia, 16%;[107] Northern Ireland, 22%;[256] Morocco, 30%;[92] Italy, 37%;[72] Ireland, 39%;[208] and France, 81%.[254] Almost all reported isolates from raw milk samples have been categorized as nonvirulent environmental strains.

According to Greenwood et al.,[89] strains of *Yersinia* could be isolated from a sample of milk taken immediately after pasteurization but before bottling, even though the time/temperature conditions applied during pasteurization appeared to be adequate. The presence of *Y. enterocolitica* has been demonstrated also in pasteurized milk in other studies.[61,62,171,227] As *Y. enterocolitica* can grow in milk at refrigeration temperatures,[230] consumption of pasteurized milk contaminated with *Yersinia* constitutes a potential health risk. However, pasteurized milk, cheese made from pasteurized milk, and milk products such as ice cream prepared from pasteurized mixes should not pose a health problem unless recontaminated with *Y. enterocolitica*, because the pasteurization process usually is adequate to destroy *Y. enterocolitica*. In the study of Greenwood et al.,[89] it also proved that the bottles into which the pasteurized milk was filled were probably a source of contamination. None of the strains found were virulent.

DeBoer et al.[61] found 4 positive samples (4.5%) of 89 Brie and Camembert cheeses, and 1 positive sample (2.0%) of 50 blue veined cheeses. None of the isolated strains proved to be virulent. In another study,[92] 7 (7.4%) of 94 cheeses were contaminated with nonvirulent *Yersinia*, all the positive cheeses being made of raw milk.

In conclusion, it is almost solely in connection with outbreaks caused by contaminated pasteurized milk,[12,88,239] reconstituted powdered milk,[170] and contaminated chocolate milk[30] (Table 49–6) that one has been able to find the pathogenic strains. If not found in the product itself, epidemiological investigations have incriminated the product. In contrast, the screening of "normal" samples has most often only revealed nonvirulent strains. Nevertheless, the consumption of raw milk or dairy products made from raw milk may pose a potential public health problem.

Seafood. Some seafoods may be consumed raw, and thus the presence of virulent strains of *Y. enterocolitica* due to fecal contamination of water or contamination during processing could cause foodborne illness. The presence of environmental strains of *Y. enterocolitica* has been reported in fish,[11,61,124,204] mussels,[226] and oysters in Canada[247] and in the US.[149] *Y. enterocolitica*-like bacteria have been isolated from shrimp and crab from the Gulf of Mexico.[197]

Vegetables. Raw vegetables might be contaminated with feces from wild animals or from the use of animal or human wastes as fertilizer, and may constitute a health risk.[61] Contamination might occur also in a food processing plant when vegetables are washed and prepared, but it should be mentioned that vegetables are usually contaminated with large numbers of other bacteria and yeasts, and *Yersinia* is a poor competitor,[230] so extensive outgrowth of the organism is unlikely. However, a single outbreak of yersiniosis was caused by pickled vegetables in China.[15]

In France, *Y. enterocolitica* has been recovered from carrots, tomatoes, and green salads from hospital foods.[152] Some isolations have been made from vegetables in Czechoslovakia.[6] Thirty (46.1%) of 65 samples in a French study,[63] and 37 (43%) of 87 samples in one study from The Netherlands[61] were reported to be *Yersinia* positive. None of the isolated strains proved to be virulent.

49.6 EPIDEMIOLOGY

49.6.1 Infection with *Y. enterocolitica*

Sporadic Cases. *Y. enterocolitica* has been isolated from humans in many countries of the world, but it seems to be found most frequently in cooler climates (North America; the western coast of South America; Europe; northern, central, and eastern Asia; Australia; New Zealand; and South Africa).[8,122,166,267,268] The widespread nature of *Y. enterocolitica* has been well documented; by the mid 1970s, Mollaret and colleagues[166] had compiled reports of isolates from 35 countries on six continents. *Y. enterocolitica* infections are an important cause of gastroenteritis in the developed world, occurring particularly as sporadic cases in northern Europe[31,58] (Table 49–5), where a clustering of cases during autumn and winter has been reported.[267]

There are appreciable geographic differences in the distribution of the different phenotypes of *Y. enterocolitica* isolated from man (Section 49.2.2 and Table 49–4).[166] There is also a strong correlation between the serovars isolated from humans and pigs in the same geographical area.[22,68,196,219,258]

Serovar O:3 is widespread in Europe, Japan, Canada, Africa, and Latin America. Sometimes, but not always, phage typing makes it possible to distinguish between European, Canadian, and Japanese strains.[128,166] Serovar O:3 seems to be responsible for more than 90% of the cases in Denmark, Norway, Sweden, and New Zealand, and as many as 78.8% of the cases in Belgium in 1994 (Table 49–5). Serovar O:9/biovar 2 is the second most common in Europe, but its distribution is uneven; while it still accounts for a relatively high percentage of the strains isolated in France, Belgium, and the Netherlands, only a few strains have been isolated in Scandinavia.[267] However, recent data show that serovar O:9 is on the decrease in Belgium. In 1979–1981, this serovar was implicated in 23.3% of the cases, while in 1994 it was responsible for only 5.9% of the cases[162] (Table 49–5). A similar reduction has been seen also in France and The Netherlands [L. de Zutter, personal communication, 1996]. Until recently, the most frequently reported serovars in the US were O:8 followed by O:5,27.[29,166,190,269] In recent years, serovar O:3 has been on the increase in the US; O:3 now accounts for the majority of sporadic *Y. enterocolitica* isolates in California.[29] In 1989, the estimated cost of yersiniosis in the US was $138 million.[269]

The first Japanese case of *Y. enterocolitica* O:8 infection was linked to consumption of imported raw pork,[108] although O:8 infections from raw water have occurred also in Japan.[99]

The incidence of *Y. enterocolitica* infection in patients with acute endemic enterocolitis ranges from 0% to 4%, depending on the geographic location, study method, and population.[129] In Australia, Canada, Denmark, Germany, New Zealand, Norway, and Sweden (Table 49–5)[8,145,176,234,272] (Danish Zoonosis Centre, Copenhagen; National Institute of Public Health (Oslo), *Y. enterocolitica* has surpassed *Shigella*, and now rivals *Salmonella* and *Campylobacter* as a cause of acute bacterial gastroenteritis. Worldwide surveillance data show great changes over the last two decades. There appears to have been a real and generalized increase in incidence,[190,267,269] even though there is still much underreporting. Nevertheless, improvements in detection and reporting systems may have contributed a great deal to the increase in reported incidence.

Only a few epidemiological studies have been performed to investigate the sources of sporadic human infections. A 1985 study of *Y. enterocolitica* in Belgium identified consumption of raw pork as a risk factor for disease.[241] The following variables were found to be independently related to an increased risk of yersiniosis in a case-control study conducted in Norway: drinking untreated water, general preference for meat to be prepared raw or rare, and frequency of consumption of pork and sausages.[192] The infrequent occurrence of infections with *Y. enterocolitica* in some areas of the world may be due in part to avoidance of certain risk factors, such as lack of consumption of pork in Muslim countries.[212] Two seroepidemiological studies have indicated that occupational exposure to pigs may be a risk factor,[159,181] although in these two studies, confounders could not be excluded. Fecal-oral transmission from person to person may probably occur.[58]

Outbreaks. In the US, chocolate milk,[30] pasteurized milk,[239] soybean curd (tofu),[238] and bean sprouts[1]

Table 49–5 Incidence of Yersiniosis in Some Countries

Country (Year)	*Cases*	*Cases per 100,000 Inhabitants*
Belgium (1994)[162]	829[‖]	8.5
Denmark (1994)[*]	643[¶]	12.4
The Netherlands (1991)[†]	115	0.8
New Zealand (1994)[272]	3,000 (estimate)[¶]	84 (estimate)
Norway (1994)[‡]	194[¶]	4.5
Sweden (1994)[234]	1,027[¶]	11.7
Switzerland (1993)[§]		1.4
United States (1989)[268]	20,000 (estimate)	8.3 (estimate)

[*] Danish Zoonosis Centre, Copenhagen

[†] H. Stegemann, personal communication, 1995

[‡] National Institute of Public Health, Oslo

[§] Swiss National Reference Laboratory for Foodborne Diseases, Berne

[‖] Serovar 0:3: 78.8%; serovar 0:9: 5.9%

[¶] Serovar 0:3: >90%

Table 49–6 Outbreaks of Foodborne Disease Due to *Y. enterocolitica*

Outbreak		*Number of Persons*		*Mode of Transportation or Vehicle*	*Serovar*	*Reference*
Year	*Place*	*Ill*	*At Risk*			
1971	Karvina, Czechoslovakia (two nursery schools)	75	142	Unknown. Common food supplies to both nurseries		189
1972	Finland (hospital)	6	—	Nosocomial	0:9	246
1972	Japan (junior high school)	198	1,086	Unknown. Presumed common source	0:3, 0:5, 0:9	273
1972	Japan (community outbreak)	189	441	Unknown	0:3	16
1972	Japan (community outbreak)	544	1,042	Unknown	0:3	16
1972	North Carolina (intrafamilial)	16	21	Sick puppies	0:8	91
1974	Japan (school)	296	814	Unknown	0:3	155
1975	Japan (school)	145	486	Unknown	0:3	155
1976	New York	222	2,193	Contaminated chocolate milk	0:8	30
1979	Japan (school)	184	954	Unknown	0:3	155
1979	Japan (institution)	6	92	Unknown	0:3	155
1980	Japan (school)	1,051	8,835	Unknown	0:3	155
1980	Newfoundland, Canada (hospital)	9	—	Nosocomial	0:5	207
1981	Canada (family outbreak)	3	4	Unknown. River water?	0:21	154
1981	Japan (school)	641	1,812	Unknown	0:3	155
1981	New York State (summer diet camp)	239	455	Reconstituted powdered milk dispenser or chow mein	0:8	170
1981–1982	Washington	44		Tofu-plant. Spring water source contaminated	0:8	238
1982	Tennessee, Arkansas, Mississippi	>172	—	Pasteurized milk	0:13,18	239
1982	Pennsylvania (Brownie troop)	16	33	Bean sprouts grown in water from contaminated well	—	1
1984	Canada	3	4	Well water	0:3	245
1984	Japan (school)	102	902	Unknown	0:3	155
1984–1985	UK (pediatric wards of hospital)	36	—	Pasteurized milk	0:10K 0:6,30	88
1985	New York (four-infant cluster)	4	4	Unknown. No obvious common exposure	0:3	137
1987	Shengyang, China (school)	351	—	Pickled vegetables	0:3	15
1988	Kristianstad, Sweden	75	—	Pasteurized milk	0:3	12
1988	Georgia (infants and children)	15	—	Household preparation of chitterlings	0:3	146
1994	Västernorrland, Sweden	13	—	Unknown, pork?*	0:3	234
1994–1995	Buffalo, New York	12	—	Pork chitterlings	0:3	136
1995	Upper Valley of Vermont, New Hampshire	10	—	Pasteurized milk	0:8	52
1995–1996	Buffalo, New York	17	—	Pork chitterlings	0:3	136

* pork is probably the vehicle (B. de Jong, personal communication, 1996)

have been implicated as sources in outbreaks of *Y. enterocolitica* infection (Table 49–6). These three outbreaks, all of which occurred before 1983, were caused by *Y. enterocolitica* serovars that have been infrequently associated with human disease (serovars O:13, O:18) or that no longer predominate in the US (serovar O:8). More recently, the preparation of raw pork intestines (chitterlings) was associated with outbreaks of *Y. enterocolitica* O:3 infections among black US infants;[136,146] the organism was isolated from samples of the pork intestines.

The milkborne outbreak in Sweden in 1988[12] was caused probably by recontamination of pasteurized milk because of lack of chlorination of the water supply. In the multistate outbreak in 1982,[239] milk cartons were contaminated with mud from a pig farm.[18] In the case of the outbreak described by Greenwood and Hooper,[88] postpasteurization contamination may have occurred from bottles. Previous studies have shown that milk-associated *Y. enterocolitica* outbreaks have been linked to the addition of ingredients after pasteurization.[30,170]

In 1981, an outbreak of infection due to *Y. enterocolitica* O:8 in Washington State occurred in association with the consumption of tofu packed in untreated spring water.[238] The outbreak serovar was isolated from the spring water samples. Another outbreak caused by serovar O:8 was traced to ingestion of contaminated water used in manufacturing or preparation of food.[217] Two other *Yersinia* outbreaks have been associated with well water. One occurred among members of a Pennsylvania girl scout troop after they ate bean sprouts grown in contaminated well water;[1] the other was a familial outbreak of yersiniosis in Canada.[245]

The epidemiology of yersiniosis in the US seems to have evolved into a pattern similar to the picture in Europe,[29,36,190] where foodborne *Yersinia* outbreaks are rare and where serovar O:3 predominates.[166,201,253] Although yersiniosis appears to be more common in Europe than in the US, only five foodborne outbreaks have been reported in Europe.[12,88,189,234,246]

49.6.2 Epidemiological Tools

Methods that have been used to differentiate pathogenic isolates of *Y. enterocolitica* include biotyping,[264] serotyping,[7,259] antibiogram typing,[20,56] phage typing,[20,167,183] and multilocus enzyme electrophoresis.[51] Methods based on the characterization of the genotype include plasmid profile analysis, restriction enzyme analysis of plasmid and chromosomal DNA (DNA fingerprinting), pulsed-field gel electrophoresis (PFGE),[44,172,211] and the use of DNA or RNA probes.[14,156,242,255]

In a comparison of six methods for typing *Y. enterocolitica*,[128] antimicrobial susceptibility had the greatest overall discriminatory power, followed by restriction enzyme analysis of chromosomal DNA (REAC), H-antigen typing, restriction enzyme analysis of plasmid DNA (REAP), phage typing, and biotyping (in decreasing order of discrimination) (Table 49–7). Antimicrobial susceptibility testing is a highly standardized method employed in a large number of laboratories; however, susceptibility patterns may be influenced by local antibiotic usage, which may make differentiation of outbreak strains from epidemiologically unrelated strains difficult. On the basis of the investigation of strains from three outbreaks, Baker and Farmer[20] concluded that even though antimicrobial susceptibility testing contributed to the differentiation of the isolates, results were less reliable than those obtained by serotyping and phage typing. Using multilocus enzyme electrophoresis to investigate allelic variations in the chromosomal genomes of the same strains would have given an overall discrimination index of 0.752, indicating the method to be less suitable than antimicrobial susceptibility for tracing epidemiologically related *Y. enterocolitica* strains.[51] The discriminatory power of the genotypic methods was greatest within serovar O:8 (Table 49–7). Because this serovar is homogeneous with regard to biovar and phagevar, there has long been a need for methods that permit a finer degree of differentiation in epidemiological investigations.[20] A bacteriophage typing system that allows greater differentiation of O:8 strains has been described.[20] The results also show that restriction enzyme analysis is an effective method for the analysis of serovar O:8. Some studies demonstrate clearly the high discriminative power of PFGE toward the differentiation of highly related *Y. enterocolitica* strains that belong to the same biovar/serovar.[70,74,97] But a comparison between PFGE and the methods used in Table 49–7 is still missing.

49.6.3 Infection with *Y. pseudotuberculosis*

Like *Y. enterocolitica*, *Y. pseudotuberculosis* is also isolated most frequently in cooler climates.[8] In the developed part of the world, *Y. pseudotuberculosis* infections do not play any significant role; however, the bacterium is found in wild animals as well as in pets.[8,9] The serovars isolated from animals are identical with those detected in humans, suggesting that the source of human *Y. pseudo-*

tuberculosis infections can be found in domestic and wild animals and birds.[8]

Because *Y. pseudotuberculosis* is so common in animals, their feces are quite likely to contaminate soil, water, and vegetables and thus contribute to transmission to man.[8,9] The view that *Y. pseudotuberculosis* may be a cause of foodborne diseases probably has been encouraged by reports of its survival on and isolation from vegetables[5] as well as the occurrence of family outbreaks.[205]

Water and soil in Japan and the far eastern regions of Russia are contaminated with *Y. pseudotuberculosis*.[78] Unchlorinated drinking water from wells, springs, and streams in mountainous areas was considered to have caused an epidemic outbreak and sporadic infections with *Y. pseudotuberculosis* in Japan.[78,79,111] Transmission of *Y. pseudotuberculosis* to humans through water contaminated by feces from wildlife and domestic animals has been reported.[76,77]

49.7 FACTORS AFFECTING SURVIVAL AND GROWTH OF *Y. ENTEROCOLITICA*, PARTICULARLY IN FOODS

Y. enterocolitica is a facultative organism able to multiply in both aerobic and anaerobic conditions.

49.7.1 Temperature

The ability of *Y. enterocolitica* to multiply at low temperatures is of considerable concern to food producers (Figure 49–1). The reported growth range is –2° C to 42° C.[23,84] Optimum temperature is 28° C to 29° C.[23] *Y. enterocolitica* can multiply in foods such as meat and milk at temperatures approaching and even below 0° C.[93,149,229,230] It is important to recognize the rate at which *Y. enterocolitica* can multiply, which is considerably greater than that for *L. monocytogenes*[27] (Table 49–8). Results show that, in a food with a neutral pH stored at 5° C, *Y. enterocolitica* counts may increase from $10\ \text{ml}^{-1}$ to $2.8 \times 10^{7}\ \text{ml}^{-1}$ in 5 days.

49.7.2 pH

The minimum pH for growth has been reported as being between 4.2 and 4.4,[132] while in a medium in which the pH had been adjusted with HCl, growth occurred at pH 4.18 and 22° C.[42] The results in Table 49–9 show the ability of *Y. enterocolitica* to multiply at pH 4.6 and give an indication of the extent to which growth occurs in 6.5% NaCl.[232]

The presence of organic acids will reduce the ability of *Y. enterocolitica* to multiply at low pH, acetic acid being more inhibitory per gram mol at a given pH than lactic and citric acids.[42]

49.7.3 Other Factors Singly and in Combination

The effect of lactic acid (concentration range of 0.1 to 1.1% v/v within a pH range of 3.9 to 5.8 at 4° C) on *Y. enterocolitica* O:9 is greater under anaerobic than aerobic conditions, although the bacterium has proved to be more tolerant of low pH conditions under anaerobic atmosphere than under an aerobic atmosphere in the absence of lactic acid.[67] *Y. enterocolitica* is not able to grow at pH <4.2 or

Table 49–7 Comparison of Discriminatory Power of *Y. enterocolitica* Typing Methods

	Serovar 0:8 (n = 16)		*Serovar 0:3 (n = 33)*		*Serovar 0:9 (n = 11)*		*Total (n = 72)*	
Typing Method	*No. of Types*	*DI**	*No. of Types*	*DI*	*No. of Types*	*DI*	*No. of Types*	*DI*
Anti-biogram	9	0.908	19	0.901	11	1.000	43	0.965
REAC†	10	0.900	4	0.623	2	0.509	22	0.903
H-antigen typing	2	0.533	6	0.608	3	0.564	16	0.840
REAP‡	6	0.808	3	0.174	1	0.000	15	0.793
Phage typing	1	0.000	3	0.443	1	0.000	7	0.770
Biotyping	1	0.000	2	0.170	1	0.000	9	0.722

* = DI, Simpson's index of diversity.[105] This index estimates the probability that two unrelated strains sampled at random from the test population will be placed into different typing groups.

† = Restriction enzyme analysis of chromosomal DNA

‡ = Restriction enzyme analysis of plasmid DNA

Source: Adapted with permission from G. Kapperud et al., Comparison of Restriction Endonuclease Analysis and Phenotypic Typing Methods for Differentiation of *Yersinia Enterocolitica* Isolates, *Journal of Clinical Microbiology*, Vol. 28, pp. 1125–1131, © 1990, American Society for Microbiology.

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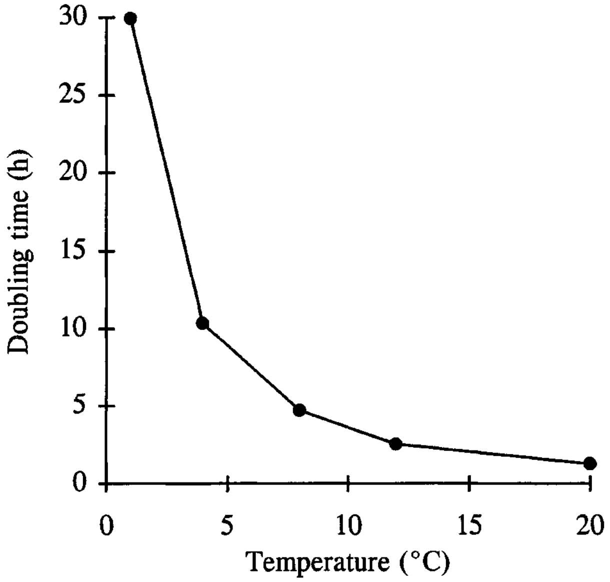


Figure 49–1 Effect of Temperature on Doubling Time. The effect of temperature on the doubling time of a mixture of three strains of *Yersinia enterocolitica*, serovar O:9 in tryptose-soy-yeast-extract-glucose medium, pH 7, in aerated culture (small volumes in shaken flasks). Courtesy of Ministry of Agriculture, Fisheries and Food, UK. *Source:* J. Plowman, H.P. Coleman, and S.M. George, unpublished, courtesy of Ministry of Agriculture, Fisheries and Food, U.K.

Table 49–8 Experimentally Determined Generation Times (GT) for *Y. enterocolitica*

Temperature (° C)	*pH*	*NaCl*	*NO_2*	*GT (h)*
5	4.5	0.5	0	22.4
5	7.5	0.5	0	5.6
19	7.5	0.5	0	1.1
19	7.5	5.0	0	1.2
19	7.5	5.0	0	1.0

Avirulent, plasmid-less strains derived from plasmid-bearing, virulent strains (serotype O:3, O:5,27, and O:8) were used because they grew faster than the corresponding virulent strains, and therefore gave a "worst-case" result.

Source: Adapted with permission from S. Bhaduri et al., Response Surface Model of the Effect of pH, Sodium Chloride and Sodium Nitrite on Growth of *Yersinia Enterocolitica* at Low Temperatures, *International Journal of Food Microbiology*, Vol. 23, pp. 333–343, © 1994, Elsevier Applied Science.

>9.0[132,229] or at salt concentrations greater than 7% (a_w <0.945).[229]

49.7.4 Growth and Survival in Foods

The ability to propagate at refrigeration temperature in vacuum-packed foods with a prolonged shelf-life[94,95] is of considerable significance in food hygiene. *Y. enterocolitica* may survive in frozen foods for long periods.[217]

The organism does not survive pasteurization or normal cooking (ie, boiling, baking, frying) temperatures. Heat-treatment of milk and meat products at 60° C for 1 to 3 min effectively inactivates *Y. enterocolitica.*[149] *D*-values determined in scalding water were 96, 27, and 11 seconds at 58° C, 60° C, and 62° C, respectively.[237]

The literature is contradictory regarding the multiplication of *Y. enterocolitica* in meat during conventional cold storage.[33,75,93,133,149,217,229,230] A comparison of published[96] and predicted generation times (GT)[232] for *Y. enterocolitica* in raw pork at 7° C, 0.5% (w/v) NaCl, and pH 5.5–6.5 shows GTs of 8.4 to 12.4 hours (published) and 8.15 to 5.05 hours (predicted). However, according to many reports, the ability of *Y. enterocolitica* to compete with other psychrotrophic organisms normally present in food may be poor.[75,133,217,230] In contrast, a number of studies have shown that *Y. enterocolitica* is able to multiply in foods kept under chill storage and might even compete successfully.[33,84,87,93,149,151,229] Pig carcasses are often held in chilling rooms for 3 to 4 days after slaughter prior to cutting. Prepackaged raw meat products may remain in retail chill cabinets for more than a week, depending on the product, packaging, package atmosphere, and rate of turnover. Pathogenic variants of *Y. enterocolitica* might multiply considerably during the course of this relatively long storage period.

As a facultative organism, the growth of *Y. enterocolitica* is drastically affected by a gaseous atmosphere. Under anaerobic conditions, *Y. enterocolitica* is unable to grow in beef at pH 5.4 to 5.8, whereas growth occurs at pH 6.0.[87] 100% CO_2 is reported to inhibit the growth of *Y. enterocolitica.*[84,164] In the study of Gill and Reichel,[84] *Y. enterocolitica* was inoculated into high pH (>6.0)

Table 49–9 Experimentally Determined Generation Times (GT) for *Y. enterocolitica*

Temperature (° C)	*pH*	*NaCl*	*NO_2*	*GT (h)*
5	5.5	2.5	0	14.76
10	6.0	6.5	0	21.18
15	7.0	0.5	0	1.67
25	4.6	0.5	0	2.15

Three strains were used, one serovar O:3 and two serovar O:5,27.

Source: Adapted with permission from J.P. Sutherland and A.J. Bayliss, Predictive Modeling of Growth of *Yersinia Enterocolitica*: The Effects of Temperature, pH and Sodium Chloride, *International Journal of Food Microbiology*, Vol. 21, pp. 197–215, © 1994, Elsevier Applied Science.

beef DFD (dark firm dry)-meat. Samples were packaged under vacuum or in an oxygen-free CO_2 atmosphere maintained at atmospheric pressure after the meat had been saturated with the gas and stored at -2° C, 0° C, 2° C, 5° C, or 10° C. In vacuum packs, *Y. enterocolitica* grew at all storage temperatures at rates similar to or faster than those of the spoilage flora. In CO_2 packs, the bacterium grew at both 5° C and 10° C, but not at lower temperatures.

Mohammad and Draughon[163] investigated the growth characteristics of *Y. enterocolitica* strains in pasteurized milk at 4° C. Pasteurized milk was inoculated with 10 or 1,000 cells ml^{-1} of *Y. enterocolitica*. *Y. enterocolitica* competed well with the background microflora and reached log numbers of 5.0 to 7.0 ml^{-1} after 7 days; however, a study of Stern et al.[230] indicated that while *Y. enterocolitica* has the capacity for growth in milk at refrigeration temperatures, it is a poor competitor with common spoilage organisms.

49.7.5 Survival and Inactivation in Water

Survival in Untreated Water. The 3-log reduction (99.9% inactivation) time for *Y. enterocolitica* in lake water has been reported to be 17 to 18 days at 4° C and 14 to 15 days at 10° C; the bacterium possibly surviving for a longer time in cold, clean surface waters than *E. coli*.[153] A Polish study described an inactivation time (T_{100}) of 38 days at spring temperatures and 7 days under summer conditions in unfiltered water, in contrast to 197 and 184 days, respectively, in filtered water.[65] Some strains of *Y. enterocolitica* are even able to grow in water at low temperatures (4° C).[102]

Inactivation in Chlorinated Water. In the study of Lund,[153] autoclaved tap water (pH 6.5) was chlorinated according to conventional water-treatment practices, resulting in a free residual level of approximately 0.05 mg l^{-1} after a contact time of 30 minutes. A 3-log reduction for *Y. enterocolitica* and *E. coli* exposed to 0.2 mg l^{-1} Cl_2 was obtained in 20 to 180 and 20 to 25 seconds, respectively, depending on bacterial strain, plasmid content (*Y. enterocolitica* O:3 harboring a 40 to 50 Mdal virulence plasmid exhibits enhanced resistance to chlorine), and temperature.[153]

Inactivation by Ultraviolet (UV) Radiation. The UV radiation dose at 254 nm required for a 3-log reduction (99.9% inactivation) of *Y. enterocolitica* O:3 and *E. coli* is 2.7 and 5.0 mWs cm^{-2}, respectively. Using *E. coli* as the basis for comparison, it appears that *Y. enterocolitica* O:3 is more sensitive to UV than many of the pathogens associated with waterborne disease outbreaks and can be inactivated readily in most commercially available UV reactors.[46] In contrast, Carlson et al.[50] demonstrated that avirulent strains were more sensitive to UV radiation (3-log dose of 7 to 10 mWs cm^{-2}) than a virulent *Y. enterocolitica* O:8 strain, which contained plasmids of 41 and 73 Mdal (3-log dose of 22 mWs cm^{-2}).

49.8 PRINCIPLES OF DETECTION AND ISOLATION

49.8.1 General Principles

To isolate *Y. enterocolitica* from foodstuffs, methods other than those used for isolation from patients with acute intestinal infection are in part required.[120,158,215,217] This is mainly due to four factors:

1. The background flora in foodstuffs varies considerably, both with regard to number and species distribution.
2. The *Yersinia* count in foods usually is far less than in clinical samples. The inclusion of an enrichment step in the isolation procedure is therefore required.
3. The bacteria may be sublethally damaged after exposure to cold, heat, or chemicals. This may necessitate a resuscitation step.
4. A broad range of different *Yersinia* is usually found in foods, and most of these strains are of no particular clinical significance.[120,149] An isolation method that as far as possible specifically selects pathogenic variants is desirable therefore.

The individual biovars and serovars of *Y. enterocolitica* differ in their tolerance to selective agents and to the conditions encountered during the isolation process. The choice of procedure will thus have a considerable influence on the range of bacteria isolated. Differences in tolerance occur between the different pathogenic serovars (O:3, O:8, and O:9).[217] A method for the isolation of pathogenic *Y. enterocolitica* strains that works well for one serovar may not necessarily be effective for another.

49.8.2 Specific Procedures for the Detection and Isolation of *Y. enterocolitica*

A three-step method based on a combination of cold enrichment in a medium with low selectivity and subsequent inoculation into a highly selective medium has been developed for the Nordic Committee on Food Analysis.[187] The method, a reference method that can be employed for the qualitative demonstration of Y. *enterocolitica* in all types of foods, comprises three steps:

1. Three hours resuscitation at 20° C to 25° C in phosphate buffered saline (pH 7.6) with 2% sorbitol and 0.15% bile salts (PSB)
2. 8 days pre-enrichment in PSB at 4° C followed by selective enrichment in a modified Rappaport broth (MRB) (4 days at 20° C to 25° C)
3. 3 weeks' cold enrichment at 4° C in PSB.

After each step, culture is carried out on cefsulodin-irgasan-novobiocin (CIN) agar, a highly selective and differential medium that is more effective than conventional enteric media for the recovery of Y. *enterocolitica*.[100] After incubation at 28° C for 18 to 20 hours, Y. *enterocolitica* appears as 0.5- to 1.0-mm-diameter colonies with a dark red "bull's eye" and transparent border. CIN agar permits the growth of all pathogenic serovars.

Wauters et al.[262] developed a method for isolation of serovars O:3 and O:9 from meat and meat products. The procedure is based on a 2- to 3-day selective enrichment period in irgasan-ticarcillin-potassium chlorate (ITC) enrichment broth at room temperature, and is therefore very time-saving compared with the method described above. Culture is carried out on modified Salmonella-Shigella agar with 1% sodium deoxycholate and 0.1% $CaCl_2$ (SSDC). Under practical conditions, the method has proved effective for the detection of serovars O:3 and O:9, but not particularly satisfactory with regard to Y. *enterocolitica* serovar O:8 and environmental strains.[262]

Both Y. *enterocolitica* and Y. *pseudotuberculosis* seem to be more tolerant of alkaline conditions than most other *Enterobacteriaceae*, and treatment of food enrichments with potassium hydroxide (KOH) may be used to selectively reduce the level of background flora.[19] Tolerance of high pH is influenced by the suspending medium, temperature, and phase of growth;[216] however, despite some improvements in the conditions provided during exposure to high pH, a method based on treatment with KOH proved inferior to selective enrichment in bile-oxalate-sorbose broth for recovering pathogenic strains of Y. *enterocolitica* from inoculated foods.[216] Elements of the methods from the Nordic Committee on Food Analysis,[187] Schiemann,[215] Wauters,[262] and KOH treatment[216] are incorporated into the International Organization for Standardization (ISO) method (ISO 10273) (Figure 49–2).[113]

Genetic probes can be used in DNA colony hybridization to demonstrate virulent Y. *enterocolitica* strains.[123,242,255] Probes based on cloned fragments that encode for virulence factors, and synthetic oligonucleotide probes based on sequence analysis of the cloned fragments, are available.[86,103,114,123,180] A study[180] in which gene probes and colony hybridization were used following isolation either by the method of Wauters et al.[262] or the Nordic Committee on Food Analysis method[187] showed that a substantial increase in the rate of detection of pathogenic Y. *enterocolitica* could be achieved. Isolation plus hybridization increased the detection rate from 16% to 38% for the method according to Wauters et al., and from 10% to 48% for the Nordic method. The results of this investigation[180] support the supposition that conventional culture methods lead to underestimation of virulent Y. *enterocolitica* in pork products.

Wren and Tabaqchali[271] developed a simple PCR assay using oligonucleotide primers based on nucleotide sequences from two independent virulence genes that readily distinguish between virulent and nonvirulent Y. *enterocolitica*. A double PCR procedure has also been developed based on a single virulence gene on the virulence plasmid.[130]

Rasmussen et al.[206] detected Y. *enterocolitica* O:3 in fecal samples and tonsil swabs from pigs using immunomagnetic separation and PCR. O:3 cells were detected after pre-enrichment, but direct detection needed further optimization of the sample preparation procedures.

Methods for the isolation of Y. *enterocolitica* from water usually involve concentration by filtration followed by procedures similar to those used for isolation from food.[113,187,215,262]

49.8.3 Specific Comments on Procedures for the Detection and Isolation of Y. *pseudotuberculosis*

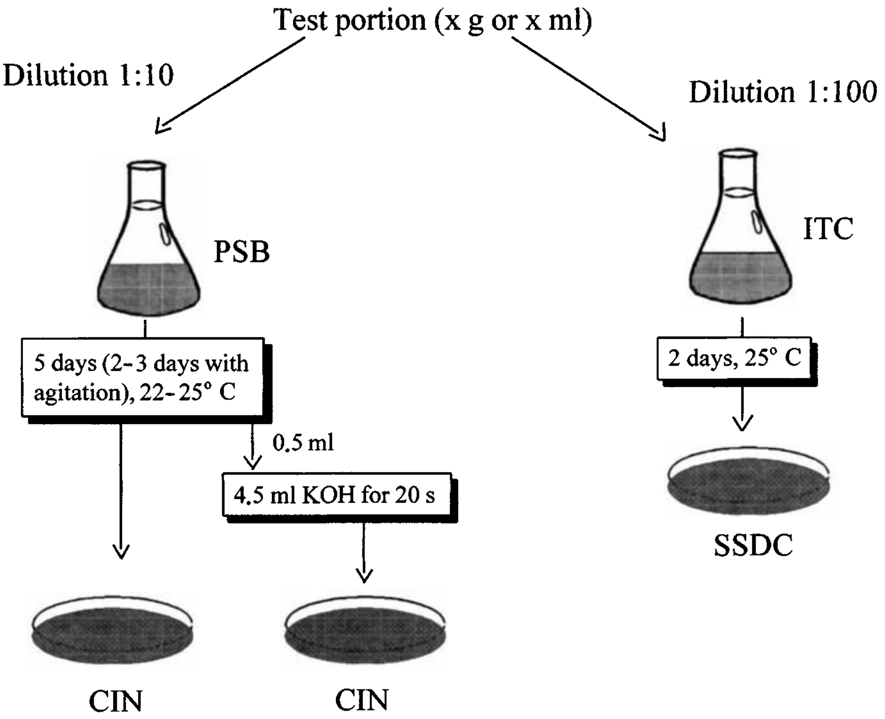
The same enrichment procedures as for Y. *enterocolitica* have proved suitable for Y. *pseudotuberculosis*,[8] and treatment of food enrichments with KOH may also be used to selectively reduce the level of background flora.[19,79]

Culture should be carried out on MacConkey agar or CIN agar. According to H. Fukushima [personal communication, 1990], cefsulodin inhibits the growth of Y. *pseudotuberculosis* at the concentrations used in CIN agar, and consequently, cefsulodin should be omitted from the medium in this case. SSDC agar is not suitable as a culture medium for Y. *pseudotuberculosis*.[8]

49.9 CONTROL

49.9.1 At the Farm Level

Newborn piglets are colonized easily and become long-term healthy carriers of Y. *enterocolitica* in the



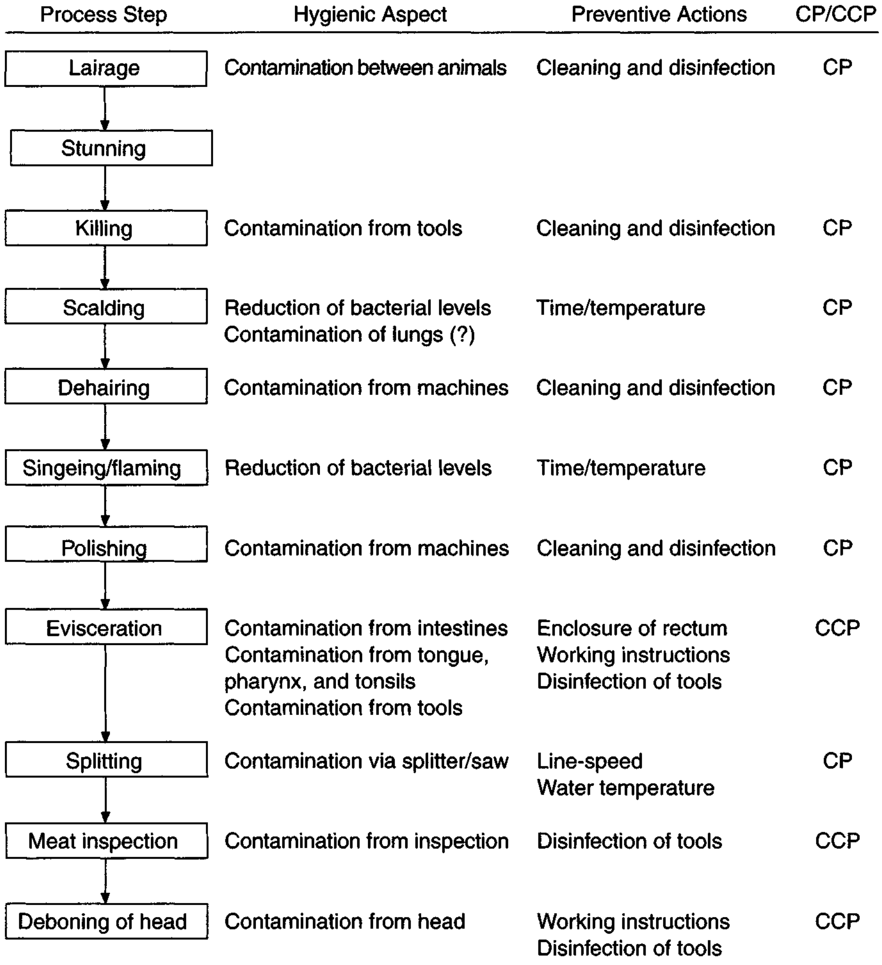
PSB = phosphate-buffered saline and sorbitol, 2%; bile salts, 0.15%. ITC = irgasan-ticarcillin-potassium chlorate enrichment broth. KOH = KOH, 0.25% (w/v); NaCl, 0.85% (w/v) in water. CIN = cefsulodin-irgasan-novobiocin agar. SSDC = Salmonella-Shigella + sodium deoxycholate, 1%; $CaCl_2$, 0.1%.

Figure 49–2 Method for Qualitative Recovery of *Y. enterocolitica* from Foods Recommended by the International Organization for Standardization (ISO)(ISO 10273).[113] The method comprises elements of the methods from the Nordic Committee on Food Analysis,[187] Schiemann,[215] Wauters,[262] and the treatment with KOH.[216]

oral cavity and intestines.[217] In a recent study,[222] an ELISA[184] was used to detect IgG antibodies against *Y. enterocolitica* O:3 in sera from 1,605 slaughter pigs from 321 different herds. Positive titers were found in 869 (54.1%) of the samples. Of the 287 herds in the final epidemiological study, 182 (63.4%) of the herds were defined as positive herds. Among the positive herds, there were significantly fewer combined herds of piglets and fatteners (53.1%) than fattening herds (88.1%). Multiple logistic regression detected several factors related to management and hygiene influencing the risk for antibody detection of *Y. enterocolitica* O:3.[222] The farm vehicles used for transport of slaughter pigs to abattoirs, daily observation of cats with kittens at the farm, and using straw for slaughter pigs increased the risk. The overall results indicate that the infection follows the live pig, and the study indicates that it is possible to establish *Y. enterocolitica* O:3 free segments of the population. Most important is to limit the contact between infected and noninfected herds by avoiding the transfer of animals from the infected to noninfected populations. Pig production is a typical pyramid structure, where a small number of herds supply the next production strata. If a successful reduction could be accomplished on the top levels of the pyramid, the results from this study suggest that a marked lowering of prevalence might be obtained in the general pig population.

49.9.2 During Slaughter

It is not possible to sort out pigs contaminated with *Y. enterocolitica* at postmortem meat inspection. Pig slaughter is an open process with many opportunities for the contamination of the pig carcass with *Y. enterocolitica*, and it does not contain any point where hazards are completely eliminated (Figure 49–3).[34] Contamination of the carcass with *Y. enterocolitica* during pig slaughter is most likely to arise from fecal and pharyngeal sources.[13,175] HACCP (Hazard Analysis Critical Control Point) and GMP (Good Manufacturing Practice) in pig slaughter must be focused on limiting this spread. As a guide, attention should be given to the possible establishment of critical points or critical control points at the following specific steps during slaughter and dressing (Figure 49–3):



Process Step	Hygienic Aspect	Preventive Actions	CP/CCP
Lairage	Contamination between animals	Cleaning and disinfection	CP
Stunning			
Killing	Contamination from tools	Cleaning and disinfection	CP
Scalding	Reduction of bacterial levels Contamination of lungs (?)	Time/temperature	CP
Dehairing	Contamination from machines	Cleaning and disinfection	CP
Singeing/flaming	Reduction of bacterial levels	Time/temperature	CP
Polishing	Contamination from machines	Cleaning and disinfection	CP
Evisceration	Contamination from intestines Contamination from tongue, pharynx, and tonsils Contamination from tools	Enclosure of rectum Working instructions Disinfection of tools	CCP
Splitting	Contamination via splitter/saw	Line-speed Water temperature	CP
Meat inspection	Contamination from inspection	Disinfection of tools	CCP
Deboning of head	Contamination from head	Working instructions Disinfection of tools	CCP

Figure 49–3 Hygienic Aspects and Preventive Actions with Respect to *Yersinia enterocolitica* during Pig Slaughter. CP, critical point; CCP, critical control point. *Source:* Adapted with permission from E. Borch, T. Nesbakken, and H. Christensen, Hazard Identification in Swine Slaughter with Respect to Foodborne Bacteria, *International Journal of Food Microbiology,* Vol. 30, pp. 9–25, 1996, © Elsevier Applied Science.

1. lairage
2. killing
3. scalding
4. dehairing
5. singeing/flaming
6. scraping/polishing
7. circumanal incision and removal of the intestines
8. excision of the tongue, pharynx, and in particular the tonsils
9. splitting
10. postmortem meat inspection procedures
11. deboning of the head.

Some of the above-mentioned critical control points will be discussed in more detail below.

During lairage, pathogenic *Y. enterocolitica* may spread from infected to noninfected pigs.[80] If possible, herds should be handled separately, and cleaning and disinfection of the lairage facilities should be performed between herds because some herds are free from this pathogen.[222]

A working procedure that is employed in many slaughterhouses, and that can be introduced immediately in all slaughterhouses, is the two-knife method. This can be used to interrupt the path of infection from oral cavity and intestine to other parts of the carcass. The two-knife method involves the installation in the slaughter hall of knife decontaminators, with running water held at a temperature of approximately 82° C. When an unclean working operation has been performed, for example, in the region around the rectum or oral cavity, the knife is rinsed before being placed in the decontaminator. The operator should then wash his or her hands before the other knife is used for clean working operations. The two-knife method should be used both by slaughtermen and meat inspection personnel.

Meat inspection procedures concerning the head also seem to represent a cross-contamination risk. Incision of the submaxillary lymph nodes is a compulsory procedure according to the regulations of the European Union (EU).[69] This may, however, result in the bacterium being transported from the tonsillar region to other parts of the carcass by the knives and hands of the meat inspection personnel.[175] In view of the fact that the incidence of tuberculosis in pigs and humans has been reduced to a very low level in many parts of the world, it may be possible to reconsider regulations that require incision of the submaxillary lymph nodes by meat inspectors.

Cutting and removal of head-meat in pigs should be carried out on a separate work table, preferably in a separate room.[69] This room should be considered to be an unclean area. Knives and equipment must not be used for cutting and deboning other parts of the carcass without prior cleaning and disinfection. Current EU regulations that require removal of pig head-meat to be carried out in a separate department are unfortunately not complied with in all abattoirs.

The possibility of decapitation early on in the carcass dressing procedure has been considered, with the head, including tongue and tonsils, then being removed on a separate line for heat treatment and cutting.[55] The results presented by Andersen[13] indicate, however, that it is even more important to modify procedures for removal of the guts to avoid contamination of the carcass from the rectum. Technological solutions have been found that allow removal of the rectum without soiling of the carcass. This can be done by insertion of a prefrozen plug into the anus prior to rectum-loosening and gut removal.[60] The sealing off of the rectum with a plastic bag immediately after it has been freed can significantly reduce the spread of *Y. enterocolitica* to pig carcasses[13,182] (Figure 49–4). According to data from the Norwegian National Institute of Public Health, the occurrence of human yersiniosis has dropped after the introduction of the plastic bag technique in about 90% of the pig slaughterhouses in Norway (Figure 49–5). In 1997, the number of reported cases in Norway was 108, corresponding to a decline of more than 40% since 1994. A similar drop in yersiniosis (about 30%) has been seen in Sweden after the introduction in this technique.[234]

49.9.3 During the Processing of Meat Products

In addition to the slaughter hall and the cutting and deboning departments, the sausage-making department must also be considered to be a contaminated area. It must be assumed that raw materials such as pig head-meat and pork cuts, and consequently also sausage meat, are likely to be contaminated with pathogenic *Y. enterocolitica*. Strict cleaning and disinfecting requirements must therefore also apply here. It is important to maintain an effective separation between sausage preparation and packing to avoid recontamination after heat treatment.

49.9.4 During Sale and Catering

Refrigeration. *Y. enterocolitica* is able to multiply at temperatures approaching 0° C. Although refrigeration of food does not prevent the multiplication of *Y. enterocolitica*, the rate at which this takes place will be reduced.

Separation of Raw Meats from Other Foods. Raw meats (in particular pork) should be separated from other foods. Cross-contamination from raw meat to heat-treated end products must be avoided in meat-processing establishments, butchers' shops, meat departments in retail food stores, and in kitchens in institutions, restaurants, and homes.

Cleaning and Disinfection. Knives, equipment, and machines used to cut or process raw meat products must be cleaned and disinfected before being used for handling other foods. All surfaces that have been in contact with raw meat must be cleaned and disinfected with appropriate and effective agents.

Adequate Cooking of Meat. A case-control study carried out in Norway revealed that inadequate heat treatment of meat is a risk factor for human yersiniosis.[192] Consumption of undercooked pork should be discouraged.

Pasteurization of Milk. Well-controlled pasteurization of milk is necessary to safeguard against *Y. enterocolitica* as well as many other pathogens.

Kitchen Hygiene. Information to the public on the consequences of eating raw and lightly cooked foods, and of the need for proper kitchen hygiene, will thus be a most important prophylactic measure. Good hygiene is particularly necessary because *Y. enterocolitica* is able to multiply at refrigeration temperature.

Personal Hygiene. Precautions aimed at preventing fecal-oral spread of the pathogens should be taken. Hand-washing and proper stool disposal must be employed in household, day-care, and hospital settings.

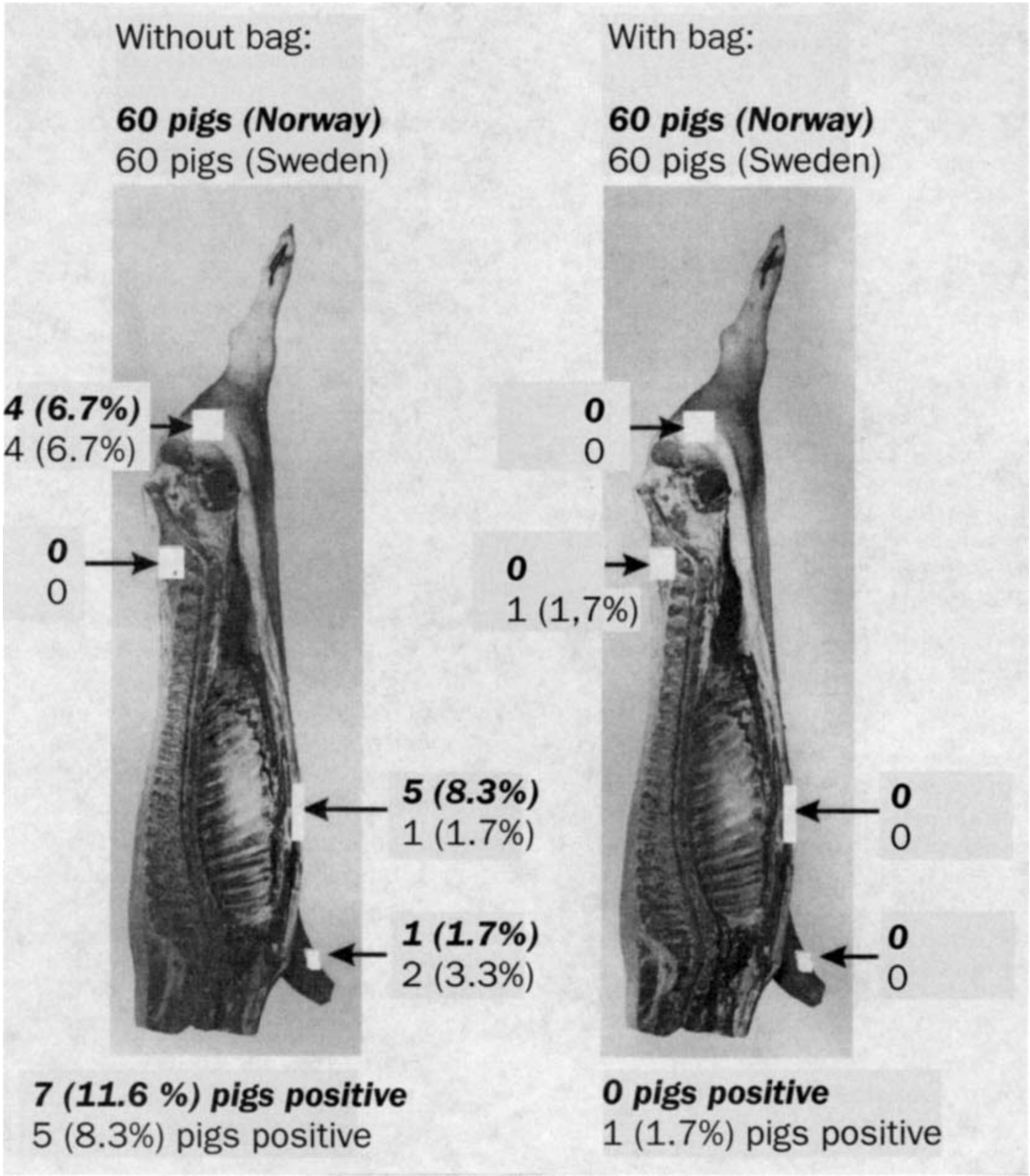


Figure 49–4 Occurrence of *Y. enterocolitica* O:3 on Pig Carcasses Eviscerated with and without a Plastic Bag To Seal Off the Rectum in one Norwegian and one Swedish Slaughterhouse. *Source:* Reprinted with permission from T. Nesbakken et al., Reduction of *Yersinia Enterocolitica* and *Listeria* spp. on Pig Carcasses by Enclosure of the Rectum during Slaughter, *International Journal of Food Microbiology,* Vol. 23, pp. 197–208. © 1994, Elsevier Applied Science.

Animal Contact. Avoidance of contact with excreta from pigs or domestic pets may reduce transmission. Domestic animals should be kept away from food preparation areas.

49.9.5 Water Supplies

Evidence discussed earlier indicates that wild and domestic animals might carry strains of *Y. enterocolitica* that are pathogenic to humans. It is therefore important not to drink from raw water supplies that are liable to contamination by animals, leakage from septic tanks, or open latrines, not to allow such water supplies to be used in food production, and to ensure that drinking water supplies are treated effectively so that *Y. enterocolitica*, and the multitude of other pathogens, are inactivated or eliminated.

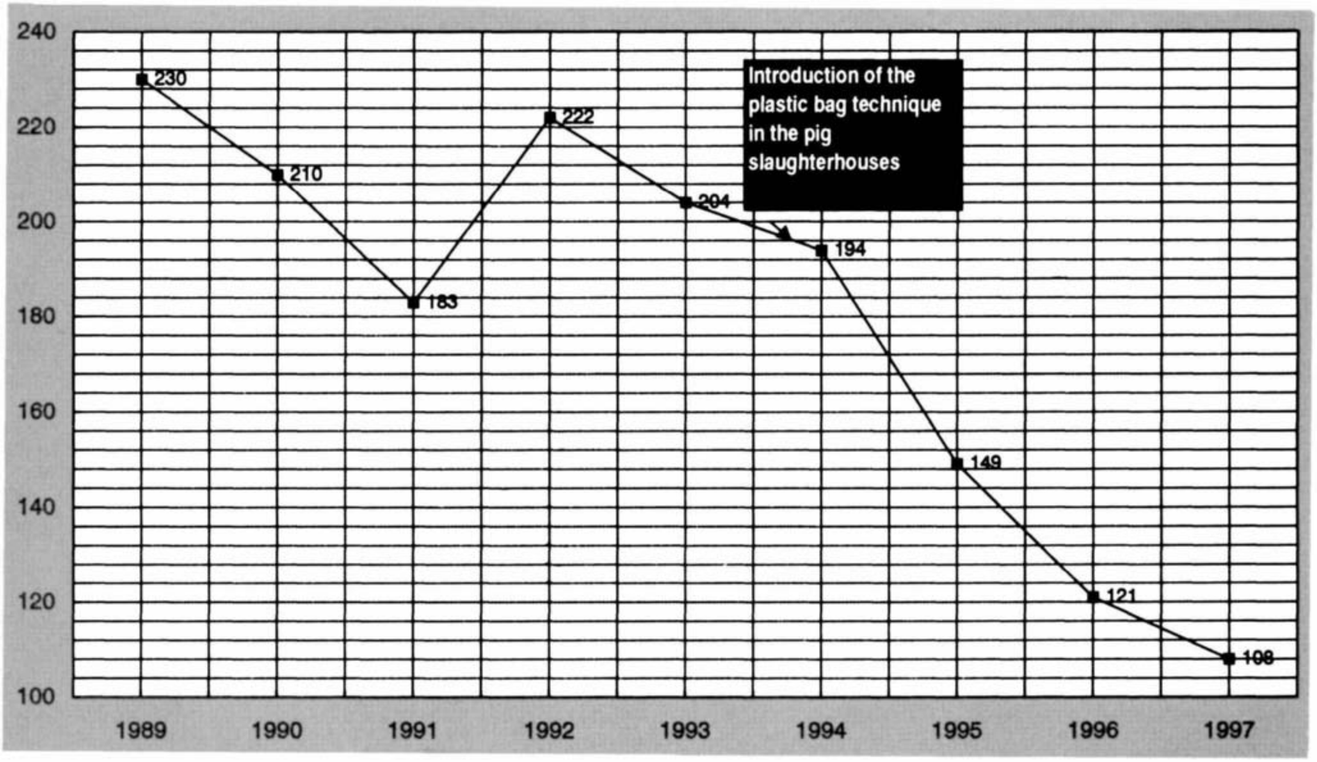


Figure 49–5. Number of Reported Cases of *Yersiniosis* in Norway. In 1994, the pig slaughterhouses in Norway started sealing off the rectum with a plastic bag. *Source:* Reprinted from National Institute for Public Health, Oslo.

49.10 CONCLUDING REMARKS

The consequences of yersiniosis are severe and include prolonged acute infections, pseudoappendicitis, and long-term sequelae such as reactive arthritis and erythema nodosum, particularly in northern Europe where the prevalence of the HLA-B27 histocompatibility type is high.[4,58,129,236,266] These consequences make *Y. enterocolitica* infection a public health and economic problem of greater magnitude than the actual number of recorded cases would suggest.[191]

The emergence of yersiniosis is related to changes that have occurred in livestock farming, food technology, and the food industry. Of greatest importance are changes in the meat industry, where meat production has shifted from small-scale slaughterhouses with limited distribution patterns to large facilities that process thousands of pigs each day and distribute their products nationally and internationally. Farm sizes have increased and animal husbandry methods have also become more intensive. While many modern slaughter techniques reduce the risk of meat contamination, opportunities for animal-to-animal transmission of the organism, and for cross-contamination of carcasses and meat products, exist on a scale that was unthinkable decades ago. In addition, advances in packaging and refrigeration now allow industry and consumers to store foods for much longer periods, a significant factor with regard to a cold-adapted pathogen such as *Y. enterocolitica*.

A Proper Understanding of the Epidemiology. Our understanding of the epidemiology of yersiniosis is still incomplete. Worldwide surveillance data show that great changes have occurred over the last two decades. The importance of *Y. enterocolitica* as the cause of a number of clinical syndromes is unclear in many areas of the world. Standardized disease surveillance is needed within and across national boundaries so that data from each location are comparable. Improved screening of stools and other specimens for *Y. enterocolitica* is necessary to further elucidate the epidemiology of the disease.

Diagnosis. The analytical methods available today for the determination of pathogenic *Y. enterocolitica* suffer from limitations such as insufficient selectivity, and, in particular, inadequate differentiation between pathogenic and nonpathogenic strains. These analytical shortcomings hopefully will be solved by the new generation of analytical techniques now being developed.

Recommended Methods of Control. At herd level, the avoidance of the purchase and introduction of animals into herds seems to be a contributing factor to the achievement of a negative carrier status.[222] The incorporation of the plastic bag technique into the slaughtering and dressing procedures by the meat industry would contribute to the prevention of the dissemination of *Y. enterocolitica* and other pathogens that spread via feces.[182] The incidence of human yersiniosis has dropped drastically since the introduction of the plastic bag technique in pig slaughterhouses in Norway and Sweden (National Institute of Public Health, Oslo).[234] HACCP and GMP in pig slaughter should focus on limiting the spread of pathogens such as *Y. enterocolitica*.

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50 Less Recognized and Suspected Foodborne Bacterial Pathogens

Michael E. Stiles

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50.1 INTRODUCTION

To separate issues of food spoilage and safety, food microbiologists have relied on a distinction between pathogenic and nonpathogenic bacteria. The microflora of foods can be divided into five categories:

1. established foodborne pathogens
2. suspected and less recognized pathogenic bacteria
3. bacteria that serve as indicators of hygiene and safety
4. bacterial agents of food spoilage
5. bacteria that are generally recognized as safe or as health promoting (probiotic) based on their use in food fermentations or as dietary adjuncts.[212]

Early associations of cases of human illness with foods centered on outbreaks of human and animal diseases such as brucellosis, diphtheria, streptococcal infections, typhoid and paratyphoid fevers, and tuberculosis. Spread of these diseases was commonly associated with foods of animal origin, particularly unpasteurized milk and dairy products and inadequately cooked or uncooked meats and fish.[25] In many parts of the world, public health measures virtually eliminated the hazard of contracting these diseases through foods and, as a result, for many years the incidence of foodborne illness was overlooked and it is still underestimated.

In the 1940s, excluding the bacteria associated with human and animal diseases, those with confirmed involvement in foodborne disease were *Clostridium botulinum*, *Salmonella* species, and *Staphylococcus aureus*.[45] These have increased from 3 to more than 15. Dack[45] listed several suspected foodborne pathogenic bacteria. Over the years some of these organisms "emerged" as foodborne pathogens, but others have remained on the list of suspected pathogens. The first detailed account of *Clostridium perfringens* was published in 1953,[117] but it remained a controversial agent of foodborne illness for 20 years before its mechanism of action was confirmed in the late 1960s. The recognition of foodborne illness due to *Bacillus cereus* was even more protracted. *Vibrio parahaemolyticus* was considered "a Japanese phenomenon" of foodborne illness until 1971 when it became recognized worldwide as a foodborne pathogen. Since the 1970s, different enteropathogenic strains of *Escherichia coli* have taken places of notoriety in foodborne illness. In the 1980s, there were many reports of "new" and "emerging" foodborne pathogens, including *Aeromonas hydrophila*, *Campylobacter jejuni* and *Campylobacter coli*, enterohemorrhagic *Escherichia coli* O157:H7, *Plesiomonas shigelloides*, various *Vibrio* species, and *Yersinia enterocolitica*.

Originally, Koch's postulates were paramount in establishing a causal role of a microorganism in disease; but these postulates cannot be proven for difficult-to-culture or nonculturable bacteria, and reliance must be placed on more sophisticated techniques[57] such as molecular techniques with PCR, DNA hybridization, and representational difference analysis. The 12 preceding chapters of this section of the book are devoted to bacteria associated with foodborne illness. The same range of bacteria was afforded separate attention in Doyle's text *Bacterial Foodborne Pathogens* published in 1989[59] except for the major change in emphasis on enterohemorrhagic *Escherichia coli* (EHEC). Since the review of "Less recognized and presumptive foodborne pathogenic bacteria" in Doyle's text,[212] the most significant development in clinical microbiology has been the change in the concept of infectious disease. In 1980, DuPont and Pickering claimed that "almost any bacterial species is capable of producing intestinal symptoms if swallowed in sufficient numbers,"[63] thereby challenging the distinction between microorganisms involved in food spoilage and food safety. This distinction is challenged further by the important change in understanding that production of disease does not reside solely with the pathogenic and virulence properties of the microorganisms but also with the health status of the host, so that "no hard and fast rule divides the human microbiota into clear-cut categories of harmless commensal organisms and pathogenic species."[126] Even lactic acid bacteria used in food fermentations have been cited as "potential pathogens."[105,217] In almost every case of one of these bacteria causing infection, there was a history of underlying disease and (or) vancomycin treatment or oral surgery.[2,87] This led Aguirre and Collins to conclude that some lactic acid bacteria are opportunistic pathogens.[2] Even this statement is speculative because conditions for this behavior, including distinguishing physiological features, must still be defined.

This conceptual change by clinical microbiologists presents a major challenge for food microbiologists, especially for safety of chill-stored and fermented foods in which large numbers of viable bacteria may be present at the time of consumption. The fact that some organisms may be facultative pathogens, depending on factors such as the virulence of the strain, the number of organisms ingested, or the susceptibility of the individual ingesting the organisms, makes a review of less recognized or suspected foodborne pathogenic bacteria more challenging. The bacteria considered in this chapter were selected for inclusion if:

- they represent "emerging" agents of foodborne disease
- they have been implicated in gastroenteritis and other human diseases, and food could serve as the vehicle for transmission
- they were recognized disease agents for which food may serve as the vehicle of transmission
- they were traditionally suspected as agents of foodborne illness but their role in disease has yet to be established.

The review takes into consideration not only the pathogens affecting the healthy population, but also those affecting the population considered to be "at risk," such as infants, young children, and the debilitated (ie, aged, hospitalized, and immunocompromised individuals).

50.2 ENTEROBACTERIACEAE

The family Enterobacteriaceae includes a large number of Gram-negative, facultatively anaerobic, rod-shaped bacteria that are motile or nonmotile and that ferment glucose, often with gas production. The name for the family was derived from *enterobacterium*, meaning an intestinal bacterium. This family is important because of the involvement of many species and strains in human and animal health. They are commonly referred to as "enteric bacteria," but this gives a restrictive impression of their distribution. They are found in soil and water, on plants, and in human and animal intestines.[74] Members of this family are important in food microbiology because they include: intestinal pathogens; the most widely used indicators of hygiene, sanitation, and food safety; and they can be important agents of food spoilage. Until the 1940s, the only recognized gastrointestinal food- or waterborne pathogens in this family were *Salmonella* spp., *Arizona hinshawii* (*Salmonella arizonae*), and *Shigella* spp. Subsequently, enteropathogenic and enterotoxigenic types of *Escherichia coli* and *Yersinia enterocolitica* have been included as gastrointestinal pathogens.[96] Based on phylogenetic criteria, *Salmonella*, *Shigella*, and *E. coli* could be included in one genus,[198] but they have been kept separate for clinical reasons. The "coliform bacteria" are lactose-fermenting members of the Enterobacteriaceae, and include *E. coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and some aerogenic *Enterobacter* species such as *E. aerogenes* and *E. cloacae*. Coliform bacteria are used as indicators of hygiene and sanitation during food processing and preparation. Many foods have "acceptable" standards for coliform bacteria. Until the 1970s, the food industry regarded *E. coli* as an innocuous indicator organism. Since then, different strains of *E. coli* have been associated with different virulence genes that have resulted in their association with an alarming array of diseases, including diarrhea, dysentery, hemorrhagic colitis, and hemolytic-uraemic syndrome (HUS), urogenitary tract infections, septicemia, pneumonia, and meningitis.[198]

In *E. coli* it is the virulence genes that make the pathogen. The five virulence types involved in enteropathogenicity of *E. coli* for humans are described in Chapter 43. Enterotoxigenic (ETEC) strains cause diarrheal illness in all age groups by elaborating cholera-like heat labile (LT-I and LT-II) and heat stable (STa and STb) enterotoxins and colonization factor antigens (CFA) I and II that enable the bacteria to adhere in the proximal small intestine. Many ETEC strains produce both CFAs, but some do not produce either, suggesting that other adherence factors can be involved. Genes for LT-I and STa are plasmid-mediated, often on the same plasmid as CFA genes. EHEC strains produce a shiga-like toxin (SLT) and cause a disease similar to shigellosis. The gene encoding SLT is on a temperate phage that could facilitate its transfer to other strains. Enteroinvasive strains cause a disease identical to shigellosis by actively invading epithelial cells of the colon, followed by cell-to-cell spread, but apparently without complications of HUS.

Members of Enterobacteriaceae not normally associated with diarrhea are referred to as "nonpathogens." This is misleading because many strains are implicated in extraintestinal disease. They may be more appropriately referred to as "opportunistic pathogens." They are a major cause of nosocomial infections that are associated with significant morbidity and mortality. Other species isolated from clinical specimens and occasionally associated with intestinal infections include *Citrobacter freundii*, *Citrobacter amalonaticus*, *Edwardsiella tarda*, and species of *Hafnia*, *Kluyvera*, *Morganella*, and *Providencia*.[77,90] Plasmid-mediated virulence traits, the relationship of their toxins to those of *Vibrio cholerae*, and their detection in other Enterobacteriaceae suggest the possibility that other genera and species also could be enteropathogenic. The organisms considered in more detail below are those suspected of being causative agents of gastroenteritis, and may be foodborne.

50.2.1 *Citrobacter*

The genus *Citrobacter* was proposed in 1932 for those "coli-aerogenes" intermediates that use citrate as the sole source of carbon. The type strain *Citrobacter freundii* is biochemically related to *Salmonella* spp., and some strains agglutinate the *Salmonella* polyvalent O antiserum. In addition to *Citrobacter koseri* (synonyms, *Citrobacter diversus* and *Levinea malonatica*) and *Citrobacter amalonaticus* (synonym, *Levinea amalonatica*), five new species and three unnamed species based on genotypic differences have been proposed.[21] *C. freundii* is a normal inhabitant of the human gastrointestinal tract, but all species of *Citrobacter* are considered to be opportunistic human pathogens. The incidence of *Citrobacter* spp. detected in the intestinal tract increases with the occurrence of diarrhea.[7,81] Early studies implicated *Citrobacter* spp. as a probable cause of diarrhea that could sometimes be traced to food handlers, but the evidence was not conclusive. *Citrobacter* spp. are found in many foods, including meat, freshwater fish, and spices.[11,200,202] Enterotoxigenic *C. freundii* may cause diarrhea, and some outbreaks have been foodborne.[77,204] Both *C. diversus* and *C. amalonaticus* cause human infection, but they are not commonly associated with diarrhea. However, in most countries, stool specimens are not routinely examined for *Citrobacter* spp., and there are no well-developed systematic procedures for selective isolation of *Citrobacter* spp. from clinical and environmental samples.[83] Isolation depends therefore on their detection from non-differentiating plating media and identification by biotyping. Simmons' citrate agar can be used as a selective medium to exclude organisms such as *E. coli*, but it does not eliminate other citrate-positive organisms. The organisms are widely distributed in soil, water, sewage, food, and in the gastrointestinal tract of humans and animals as normal inhabitants.[83]

Strains of *C. freundii* have been found that produce ST and LT toxins with high homology to cholera and *E. coli* toxins; however, there was no accumulation of fluid when these strains were tested in the rabbit ileal loop test.[132] In a separate study,[103] *C. freundii* producing STa was detected in strains isolated from children with diarrhea. Strains of *C. freundii* from beef samples and stool samples of patients with diarrhea were shown to harbor SLT-II-related genes.[202] The strains were shown to have moderate cytotoxicity and the results of the study illustrated the intergeneric occurrence of SLT-II toxins. Diarrhea due to *Citrobacter* spp. is not common and, similar to *E. coli,* strains may become pathogenic by acquiring plasmids for enterotoxin production, colonization factors, or other pathogenic mechanisms. In 1973, Sedlák[204] reviewed the genus *Citrobacter* and cited five outbreaks with up to four cases of foodborne illness in each outbreak. Meat products were implicated in two of the outbreaks; milk in another. The organisms were described as being "conditionally pathogenic bacteria." In 1995, an outbreak of severe gastroenteritis with cases of HUS occurred in a nursery school and kindergarten in Germany.[223] Verotoxigenic *C. freundii* on parsley used to prepare green butter for sandwiches was implicated as the cause of infection. The parsley had been fertilized with pig manure. Identical strains of *C. freundii* were isolated from the infected children and the parsley.[223]

50.2.2 *Klebsiella*

Klebsiella pneumoniae is an opportunistic pathogen of humans and animals. With the acquisition of antibiotic resistance, *K. pneumoniae* has become an important nosocomial pathogen.[90] It is present in the gastrointestinal tract of humans and animals, but in lower numbers than *E. coli,* so that enrichment and selective strategies are required to detect them. Environmental isolates are considered to be as "pathogenic" as human and animal strains.[14] The same applies to "fecal" and "nonfecal" isolates. DNA hybridization studies of clinical and nonclinical *Klebsiella* strains[240] indicated three groupings: *K. pneumoniae sensu lato* that includes clinical isolates and relates to a fecal coliform-positive reference organism; other *Klebsiella* "species" that include isolates of nonclinical origin and relate to a nonfecal coliform reference organism; and indole-producing *Klebsiella oxytoca* that are found in both environments. This indicates that fecal-type klebsiellae and *K. oxytoca* are most commonly associated with pathogenicity. Most *Klebsiella* spp. associated with disease and sewage are "fecal coliforms," based on their ability to grow and produce gas at elevated incubation temperatures.[13] The extent to which humans carry *Klebsiella* spp. in their intestines is not clear, but human colonization is considered a major reservoir of *K. pneumoniae* and a step toward infection.[170] Colonization requires doses in excess of 10^8 organisms for some individuals.[170] Person-to-person spread is the most common mode of transmission of *Klebsiella* spp. in hospital infections,[127] but foods have been implicated in intestinal colonization.[171] *Klebsiella* spp. can affect humans of all ages, but infants, the elderly, and immunocompromised individuals are at greatest risk.[114]

Both *K. pneumoniae* and *K. oxytoca* have been implicated in outbreaks of diarrhea.[77,143] Some strains of *K. pneumoniae* isolated from feces of people with diarrhea are enterotoxigenic, and strains that produce LT enterotoxin have been implicated in outbreaks of foodborne illness in Sweden, in which watery diarrhea was the dominant symptom.[47] ST enterotoxin produced by *K. pneumoniae* has been purified and it has cross-reactivity with, and the same potency as, *E. coli* ST enterotoxin in the suckling mouse assay.[142] The transfer of CFA/I gene from *E. coli* can occur, producing a functional adherence factor for the pathogenicity of *Klebsiella*.[243] Virulence of *K. pneumoniae* is influenced by adherence mechanisms that promote attachment of the bacteria to the epithelial cells and enable the organism to overcome the host defense mechanisms.[114] The presence of a cell capsule also is involved in virulence of *K. pneumoniae* acting to protect the cell from phagocytosis.[56,114]

K. pneumoniae has been isolated from meats, milk and dairy products, fresh vegetables and fruits, soil, dust, air and water, the hospital environment, industrial effluents, and environments not associated with fecal contamination.[13,101,170,171,197] *K. pneumoniae* was isolated from 53 of 115 vegetable salads prepared in a hospital, with counts in 37 of the salads between 10^3 and 10^6 cfu g^{-1} (colony-forming units per gram).[241] Samples of high-calorie milkshakes contained 10^3 to 10^6 ml^{-1} *K. pneumoniae*;[171] the source of contamination was traced to the milkshake mixer. *K. pneumoniae* can grow in Mozzarella cheese with only moderate temperature abuse.[160] The organism has also been reported as a pathogen of freshwater fish.[11] Its ability to produce enterotoxins and its isolation from many foods suggest a role for *K. pneumoniae* in foodborne illness.[143,170]

Foodborne outbreaks of gastroenteritis due to *K. pneumoniae* may not be reported because the organism is not considered seriously as a cause of foodborne illness in normal healthy individuals, nor has it been systematically sought as a foodborne enteropathogen. An outbreak of foodborne illness attributed to *Klebsiella* spp. was described by Horváth et al.[119] A total of 190 people became ill. Symptoms of foodborne illness appeared 10 to 15 hours after a common meal was eaten. The symptoms included headache, dizziness, vomiting, and watery diarrhea accompanied by severe cramps. There were no reports of fever, and recovery occurred in 6 to 8 hours. *Klebsiella* spp. were isolated from stool specimens, and 10^6 to 10^7 cells g^{-1} were isolated from some of the foods. Unfortunately, the isolates were not serotyped and other potential pathogens also were isolated in relatively low numbers from some of the foods. Studies in Mexico led Mesta et al.[168] to conclude that *K. pneumoniae* is a primary cause of gastroenteritis of uncompromised hosts. *K. pneumoniae* was cited by Madden et al. as an agent of foodborne illness in their review of virulence assessment of foodborne microbes.[155]

50.2.3 *Enterobacter*

The genus *Enterobacter* was recognized in the 1970s, and eight species are now distinguished.[77] *E. cloacae* and *E. aerogenes* are established species of this genus, and *E. cloacae* is the type species. *Enterobacter agglomerans* is a heterogeneous species that has been moved to a new genus, *Pantoea*.[88] There are no isolation media that are specific for *Enterobacter*. The genus is a large, heterogeneous group of organisms that are differentiated from one another and from *K. pneumoniae* by their lysine- and ornithine decarboxylase and arginine dihydrolase activities, as well as motility.[99,136] *Enterobacter* spp. normally exist as saprophytes in the gastrointestinal tract of humans and animals. They also are widely distributed in nature, in water, sewage and soil, on plants and in foods—notably dairy products, meats, vegetable salads, and spices[96,200,241]—and infant formula.[154,189] Although they are enteric bacteria, they are seldom reported as enteric pathogens; however, they are opportunistic pathogens that cause extraenteral infections, generally of nosocomial origin.[99,136] The incidence of *E. cloacae* in human infections is greater than that of *E. aerogenes*, but *E. aerogenes* might have been misidentified as *K. pneumoniae* or *E. cloacae* in earlier studies.[99] *E. sakazakii* (previously, yellow pigmented *E. cloacae*) is usually a commensal of the human intestine, but it has occasionally been isolated as an extraenteral pathogen.[99] It was also isolated from powdered milk[75] where it was reported to cause foodborne illness of infants in a hospital setting where the infection was caused by cross-contamination and temperature abuse.[178,189] The thermal resistance of *E. sakazakii* appears to be higher than that of many other Enterobacteriaceae.[177]

There were a few early reports of the "cloacae-aerogenes" group in foodborne diarrhea, but these were not substantiated. *E. cloacae* has been isolated from the small intestine of people with acute diarrhea.[143] An *E. cloacae* isolate that caused fluid secretion in a rabbit ileal loop test elaborated ST enterotoxin comparable to that from strains of *E. coli* and *K. pneumoniae*.[141] *Enterobacter* spp., especially *E. cloacae* and *E. sakazakii*, should be viewed as potential agents of foodborne illness, but more epidemiological information on these organisms is required to allow a more reliable assessment of their potential as foodborne pathogens.

50.2.4 *Hafnia*

The organism previously known as *Enterobacter hafniae* or *Enterobacter alvei* was reclassified as *Hafnia alvei*.[197] The role of *H. alvei* in gastroenteritis has been controversial, but epidemiologic evidence indicates an association between strains of *H. alvei* and diarrhea.[3,192] *H. alvei* also has been associated with extraintestinal infections, usually in patients with underlying illness; however, most *H. alvei* strains are not considered to be pathogenic for humans.[104] No special media have been developed to enrich or isolate *H. alvei*. The organism grows well on desoxycholate-lactose-sucrose-sorbitol agar.[197] It may not grow well on media typically used to isolate salmonellae, but when it does, it can resemble *Salmonella* because it does not ferment lactose. *H. alvei* generally grows well in selenite and tetrathionate broths, and its biochemical reactions are close to those of *Enterobacter* and *Serratia*.[197]

50.2.5 Other Enterobacteriaceae

Edwardsiella tarda is a well-recognized cause of extraintestinal infections in humans, but it is also considered to be a possible cause of diarrhea,[35,136,203] especially in developing countries.[196] *E. tarda* is found rarely in the feces of healthy people, but a higher isolation rate has been found among patients with diarrhea.[92] The possibility that *E. tarda* is involved in the pathogenesis of amoebic dysentery has been suggested.[92] Heat-stable enterotoxin was detected in *E. tarda* isolates.[19] Because *E. tarda* would be expected to have an enteroinvasive pathogenesis, the role of ST enterotoxin is not clear. *E. tarda* has been isolated from cold-blooded animals, which may serve as a reservoir for human infection.[196] However, the association of *E. tarda* with diarrhea does not necessarily indicate a causal relationship,[92] and food is considered an unlikely vehicle for this organism. *Kluyvera* spp. are normally regarded as saprophytes; however, they have been isolated from cancer patients and from a healthy subject with diarrhea.[72] Based on the fact that *Kluyvera* spp. can colonize the intestinal tract and they are found in food, these organisms have the potential to cause diarrhea that is foodborne in origin. It has not yet been shown that *Kluyvera* spp. cause gastroenteritis or foodborne illness.

Proteus, *Providencia*, and *Morganella* are differentiated from other members of Enterobacteriaceae by their oxidative deamination of a wide range of amino acids. Members of the *Proteus* group have long been recognized by bacteriologists because they swarm on the surface of agar media, produce a characteristic odor, and cause off-odors and spoilage of foods. Swarming is not a characteristic limited to these bacteria; in fact, it has been associated with *Salmonella* that are exceptionally virulent.[90] The genus *Proteus* has long been suspected as an agent of foodborne illness.[45] In eastern Europe investigators reported outbreaks of foodborne illness due to *Proteus* based on large numbers of organisms in implicated foods and in stools of patients with gastroenteritis.[7] The genus *Morganella* contains the organism previously known as *Proteus morganii*.[77] It was originally isolated from infants with diarrhea, and it has subsequently been implicated as a cause of diarrhea.[187] It is included with those bacteria responsible for causing histamine poisoning associated with eating fish of the suborder Scombroidea (tuna, mackerel, and bonito). These fish contain high levels of histidine, which is decarboxylated by the bacteria to histamine, saurine, and related products. The genus *Providencia* includes pathogens of the urinary tract,[77,187] for which fecal colonization serves as a potential reservoir.[113] *Providencia alcalifaciens* (previously *Proteus inconstans* biogroup A) has become a noted infectious agent of humans, particularly of the gastrointestinal tract,[187] but its causative role in gastroenteritis has not been proven.[77] Higher frequencies of isolation of *Providencia* from patients with diarrhea than for healthy persons have been reported.[7] The habitat of *Providencia* includes decaying organic matter, polluted water, soil, manure, and feces.[187] The genus *Proteus* is commonly isolated from the human intestine, and the intestine is considered a potential reservoir for infection, especially in hospitalized patients. *Moellerella wisconsensis* was isolated from feces and, in one notable instance, was implicated in a severe case of bloody diarrhea, fever, abdominal pain, and cramps contracted during overseas travel.[77] There is no evidence that *M. wisconsensis* actually causes diarrhea, but the same applies to members of the tribe *Proteeae* in general. There is no evidence that this group of bacteria has a role in foodborne illness; however, they are clinically important bacteria, primarily causing severe urinary tract infections, and because of the extreme antibiotic resistance of some strains.[187]

In the eighth edition of *Bergey's Manual*, the genus *Serratia* consisted of one species, *Serratia marcescens*. Since then, *Enterobacter liquefaciens* has been reclassified as *Serratia liquefaciens* and six other species have been added, including the atypical species *Serratia fonticola*.[77] *S. liquefaciens* occurs among the psychrotrophic microflora of meat, milk, and vegetable salads.[131,214,241] In recent years, *Serratia* have been added to the list of Enterobacteriaceae that potentially are pathogenic for humans. *S. marcescens* is recognized as a frequent cause of severe extraintestinal infections, particularly nosocomial infections.[76] At this time, available evidence does not implicate *Serratia* species as gastrointestinal pathogens or as pathogenic agents of foodborne origin;[100] however, in a study of enterotoxigenic bacteria isolated from foods, LT enterotoxin-producing strains of *S. liquefaciens* and *Serratia rubidaea* were reported.[129]

50.3 SPIRAL BACTERIA OF THE HUMAN STOMACH

Spiral-shaped bacteria traditionally were associated with the family Vibrionaceae that included four genera, of which *Aeromonas*, *Plesiomonas*, and *Vibrio* contained species associated with foodborne disease. The genus *Vibrio* still contains more than 25 species, many of which have been implicated as human and animal pathogens—especially *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Chapter 48). The group of bacteria that were known as "related vibrios" and associated with diarrhea in humans became part of the new genus *Campylobacter* and included the intestinal pathogens *Campylobacter jejuni* and *Campylobacter coli* (Chapter 40). Transmission of *C. jejuni* to humans has occurred through contaminated water, unpasteurized or recontaminated pasteurized milk, and undercooked meats, especially poultry.[233] *C. jejuni* also is transmitted to humans from pets. The genera *Campylobacter* and *Arcobacter* were proposed as the family *Campylobacteriaceae.*[176] These organisms are biochemically inert, resulting in generalized groupings such as gastric campylobacter-like organisms and urease-positive thermophilic campylobacters. The genus *Campylobacter* became a depository for varied groups of organisms. Many other species (groups) of *Campylobacter* have been described, including *Campylobacter pylori* and aerotolerant campylobacter-like strains. These became the genera *Helicobacter* and *Arcobacter*, respectively, and the family *Proteobacteria* has been described to include *Arcobacter, Campylobacter,* and *Helicobacter.*[226]

50.3.1 *Helicobacter*

Spirochetes and spirillum-shaped bacteria have been associated for many years with the stomachs of humans and animals. From the beginning of the 20th century, spiral-shaped bacteria were reported in patients with gastric cancer and peptic ulcer disease.[61] Based on 16S rRNA gene sequence analysis, the new genus *Helicobacter* was proposed, which is closely related to the genera *Campylobacter, Flexispira,* and *Wolinella*. The genus *Helicobacter* is widely distributed as intestinal bacteria in different animals. *H. pylori* is the most common cause of chronic active gastritis, and *H. fennelliae, H. cinaedi,* and *Flexispira rappini* are associated with enteritis.[94]

H. pylori is morphologically similar to *C. jejuni*, hence it was originally classified as the "pyloric campylobacter" and subsequently as *C. pyloridis* and *C. pylori.* Despite reports over a period of 20 years that campylobacter-like bacteria are associated with gastritis and gastric and duodenal ulcers, the possibility that peptic ulcer or gastric cancer might be associated with an infectious agent was discounted.[156,157] However, by 1994 a conference of the US National Institutes of Health presented a consensus regarding the role of *H. pylori* in gastric and duodenal ulcers.[180] *H. pylori* had been associated also with the chain of events leading to adenocarcinoma and lymphoma of the stomach.[61] The Consensus Conference noted that "virtually all *H. pylori*-positive patients demonstrate antral gastritis" but only a small proportion of individuals harboring the organism develop ulcers. They concluded that recurrence of duodenal ulcer can be prevented by elimination of *H. pylori* from the stomach and duodenum, but that the relationship between *H. pylori* and gastric cancer requires further study.[180] People infected with *H. pylori* have an increased risk of developing gastric adenocarcinoma and lymphoma.[180]

H. pylori is a Gram-negative, microaerophilic, motile, spiral-shaped bacterium with one to six polar flagella. An infective but nonculturable coccoid phase that reverts to the viable form has been reported.[233] *H. pylori* colonizes the nonacid-secreting mucosa of the stomach. It is considered to be a pathogen because its presence is associated with chronic active gastritis and its eradication results in resolution of the gastritis. It is present in 95% of duodenal ulcers and in 70% to 80% of gastric ulcers, but whether or not it is a frank or opportunistic pathogen is not clear.[94] Most infections are without clinical consequence and family members of patients may be asymptomatic carriers. Studies of seropositivity indicate that *H. pylori* is distributed widely and is related to socioeconomic status. Risk factors associated with gastritis include smoking, alcoholism, immunosuppression associated with HIV, and consumption of salty, smoked, or pickled foods.[18] In 1996, Wesley reviewed the risk of contracting *Helicobacter* and *Arcobacter* through foods and beverages.[233] The information is not definitive, but it appears that these infections can occur by the fecal-oral route, person-to-person through saliva, pet-to-human, and from ingestion of foods and beverages; whereas Harris and Misiewicz[106] noted that *H. pylori* has not been isolated from the environment or animal sources and they do not cite food as a possible source of infection. In any event, appropriate preparation of cooked foods should kill contaminating bacteria.

50.3.2 *Arcobacter*

Arcobacter was originally described as an aerotolerant campylobacter, *C. cryaerophila.* Aerotolerance, growth at 15° C and optimum growth at 30° C, failure to produce H_2S, and growth in the presence of 2.5% NaCl distinguish *Arcobacter* from *Campylobacter* spp.[244] *Arcobacter* infections in humans are associated with gastroenteritis. Three species have been implicated: primarily *A. butzleri*, and also *A. cryaerophilus* and *A. skirrowii.* The transmission of *A. butzleri* is associated with consumption of contaminated water or food and also person-to-person contact. The association of *A. butzleri* with pork and poultry is strongly indicated.[37,244] *Arcobacter* infections of animals are associated with abortion and enteritis, but only enteritis has been reported for human infections.

50.4 SPOREFORMING BACTERIA

The endospore-forming bacteria, primarily of the genera *Bacillus* and *Clostridium*, survive in nonsterile foods because of the resistance of spores to heat and other adverse environmental conditions. This is well-recognized in the role of *Bacillus cereus*, *Clostridium botulinum*, and *Clostridium perfringens* in foodborne illness reviewed in Chapters 39, 41, and 42, respectively.

Diarrheal illness caused by foodborne bacilli was reported early in the 1900s but the causative organisms were described as belonging to the "subtilis-mesentericus group" or called "pseudoanthrax or anthracoid bacilli." In 1948, Hauge established that it was *Bacillus cereus* that caused an outbreak of foodborne disease in Norway.[112] Nonetheless, except for *Bacillus anthracis*, members of the genus *Bacillus* were generally considered nonpathogenic until the 1960s. The genus *Clostridium* has been more traditionally associated with pathogenicity.[5] These organisms are ubiquitous, occurring in air, soil, water, feces, milk, and other foods. *Bacillus* species are facultative anaerobes, generally catalase-positive and motile; *Clostridium* species are generally obligate anaerobes and catalase-negative, and they are either fermentative or proteolytic, or both.

50.4.1 *Bacillus* Species

Despite the marked difference in significance of *Bacillus anthracis* compared with other *Bacillus* spp., it is closely related to *B. cereus*, *B. cereus* var. *mycoides*, and *B. thuringiensis*;[195] however, avirulent strains of *B. anthracis* clearly are identifiable as anthrax bacilli and not *B. cereus*.[29] *B. cereus* and *Bacillus subtilis* emerged as causative agents of foodborne illness (see Chapter 39) and as clinical and veterinary pathogens.[147] *Bacillus brevis* and *Bacillus licheniformis* are suspected as possible agents of foodborne illness. Other *Bacillus* spp. have less epidemiological evidence to implicate them as causes of foodborne illness, but their repeated occurrence in foods associated with foodborne illness has created suspicion about their involvement.[147] The symptoms of foodborne illness attributed to the less recognized *Bacillus* spp. include vomiting and diarrhea, and in some cases abdominal cramps, nausea, and pyrexia.[147] Some exceptionally short incubation periods (15 to 30 minutes) have been reported, but in general, others range from 1.5 to 10 hours. In outbreaks of foodborne illness attributed to the other *Bacillus* spp., a notable feature is the presence of the organism at high levels ($>10^6$ cfu g^{-1}) and in almost pure culture.[147,222] Suspected foods include meats, pizza, and whole meal bread.[147,222]

B. licheniformis has been implicated in incidents of foodborne illness with symptoms similar to those of *C. perfringens* food poisoning.[147] The time for onset of symptoms was 4 to 15 hours. Foods implicated were cooked meat dishes that contained *B. licheniformis* at 10^5 to 10^9 cfu g^{-1}.[128,147] *B. brevis* has been isolated from suspected foods in outbreaks of foodborne illness. The organism has been isolated also from a patient's feces.[89,147] An outbreak of *B. brevis* food poisoning was reported in 1984.[232] The mean incubation time was 4 hours and symptoms included nausea, vomiting, and abdominal discomfort. A heat-labile enterotoxin produced by the strain of *B. brevis* was responsible. There do not appear to be any special circumstances that differentiate the control of these *Bacillus* spp. from those for *B. cereus*.

50.4.2 *Clostridium* Species

Clostridium perfringens type C is a common pathogen of animals, especially young animals because of the poorly developed intestinal microflora. In humans, this organism causes a rare but severe necrotizing disease of the small bowel known as "enteritis necroticans" or "pig-bel." The possible involvement of *C. perfringens* type C in neonatal necrotizing enteritis has increased the interest in this organism.[140] A classic report of *C. perfringens* type C enteritis necroticans was written by Murrell and coworkers.[175] The disease was associated with ceremonial pig feasts in Papua, New Guinea. The organism is either part of the normal intestinal flora, or it is ingested with contaminated pork during a pig feast. Several factors are involved in the pathogenesis of this disease: overeating, poor level of nutrition, and a diet rich in trypsin inhibitors.[228] The organism proliferates in the small intestine and produces b-toxin. The toxin causes inflammation, hemorrhage, and gangrenous necrosis of the intestine.[5] The disease was reported also in Germany after World War II, where it was known as *darmbrand*, literally meaning "burning intestine."[245]

Neonatal necrotizing enterocolitis is a closely related disease, with varied causes that may include *C. perfringens* types A and C[229] and *Clostridium butyricum*.[122] Most infants who developed this disease were fed cow's milk formula rather than breast milk.[145] A case of necrotizing enterocolitis in a full-term infant was attributed to feeding concentrated formula instead of a "ready-to-feed" formula.[179] The pathogenesis of clostridial necrotizing enterocolitis has yet to be clarified;[146] however, it is hypothesized that in the neonate the disease occurs by coincidence of two of three pathological events: deficiency of blood supply to the intestine (intestinal ischaemia), colonization by pathogenic bacteria, and excess protein substrate in the intestine.[145]

Clostridium difficile is a highly infectious organism that has been implicated as a major cause (50% to 95%) of antibiotic-associated diarrhea and 20% of cases of pseudo-membranous colitis (necrotizing enterocolitis).[5,9,80] Transmission is primarily by hands of hospital workers and not by food or other environmental sources;[80] nonetheless, the diseases are considered here for their potential food or diet association. *C. difficile* may be a normal constituent of the fecal flora of healthy infants;[65] it is rare and transient in the intestine of the healthy adult[9] and it has been isolated from marine sediments, soil, hospital environment, and animal feces.[208] *C. difficile* is nonhemolytic and nonproteolytic, and it produces a wide range of fermentation products. Most strains are toxigenic, producing an enterotoxin (toxin A) and a cytotoxin (toxin B).[5] Diagnosis depends on detecting either the toxin(s) or the organism in stools, or by antibody assay.[9] Symptoms vary from serious, sometimes lethal, bloody diarrhea indicative of colitis to self-limiting, watery stools.[9] Pseudomembranous colitis occurs mainly as a result of the disruption of the normal ecology of the gastrointestinal tract caused by antibiotic therapy or drastic dietary change. *C. difficile* may be naturally present in the intestinal flora and elimination of the competing microflora allows it to grow and produce entero- and cytotoxins.[216] This organism has emerged as an important cause of mild to severe gastroenteritis, but it appears unlikely, at this time, that food plays a role in the transmission or spread of the disease. The literature on this organism and its pathogenesis is expanding rapidly.[80]

50.5 LACTIC ACID BACTERIA

Lactic acid bacteria are a loosely defined group of coccus- and rod-shaped, Gram-positive, catalase-negative bacteria that produce lactic acid as the principal end product of growth. Many of the lactic acid bacteria are commonly found in foods and used in food fermentations. The lactic acid bacteria include the genera *Aerococcus*, *Enterococcus*, and *Streptococcus*, which contain species that are frank pathogens. Phylogenetically, the lactic acid bacteria belong to the clostridial branch of the Gram-positive bacteria. They have a guanidine plus cytosine (G+C) content of their DNA that generally is less than 50 mol%. Lactic acid bacteria used as starter cultures in fermented foods or that grow as the adventitious microflora of foods are considered innocuous, in some cases, even advantageous for human health (probiotic). Lactic acid bacteria are also common commensals of the indigenous microflora of the intestinal tract and mucous membranes of humans and animals.

50.5.1 *Streptococcus*

The original genus *Streptococcus* included a wide range of bacteria. Some species produce specific carbohydrate antigens (C substances) in the cell wall, which formed the basis of the Lancefield classification system. Based on DNA homology studies, the genus was separated into three genetically distinct groups: *Streptococcus sensu stricto*, *Enterococcus*, and *Lactococcus*.[201] The species remaining in the genus *Streptococcus* include the pathogens *S. pyogenes*, *S. pneumoniae*, and *S. agalactiae*; the oral streptococci (or Sherman's Viridans group); and the nonpathogenic starter organism *S. thermophilus*. The pathogenic streptococci are β-hemolytic. *S. pyogenes* has re-emerged as a serious human pathogen with involvement in toxic shock syndrome and necrotizing fasciitis, though it appears that there may not be an increase in real incidence or severity of infections. The route of infection is from localized trauma and not from symptomatic pharyngitis; therefore, it is not likely to be from foods.[211] Streptococci can be transmitted through foods by an infected worker handling ready-to-eat foods. Before the widespread use of pasteurization and refrigeration of milk, there were frequent milkborne outbreaks of scarlet fever and septic sore throat caused by group A streptococci. Meat handling also was implicated as a means of transmission. Foodborne outbreaks were characterized by their "explosive nature." More recent outbreaks have implicated a range of prepared, ready-to-eat foods, such as egg, potato, and shrimp salads, mousse desserts, and a rice dressing.[51,159]

Closely related β-hemolytic groups B and C streptococci have been implicated also in foodborne disease, despite the fact that they may be primarily animal pathogens. *S. agalactiae* (group B) is a principal cause of bovine mastitis and a bacterial pathogen in newborn infants. Group B streptococci are found in 25% of genital and gastrointestinal tracts of human adults, and they are often carried pharyngeally.[70] The most likely source of neonatal *S. agalactiae* infections is the natural microflora of the urogenital tract. This organism only rarely has been associated with foodborne outbreaks of diarrhea; for example, breast milk fed to infants,[181] which suggests a need for extreme care in human milk banking. *Streptococcus zooepidemicus* has been implicated in outbreaks of human pharyngitis, followed by a nephritic sequela.[15,70] These outbreaks were attributed to consumption of unpasteurized milk and Queso Blanco cheese made from raw cow's milk. Group F streptococci are not widely recognized as human pathogens because they seldom infect healthy individuals, and infections are usually associated with underlying disease. However, they have been implicated in severe infections and they are unique among the streptococci for their involvement in abscess formation. Their mode of transmission is not clear, but the gastrointestinal tract is the most likely source of infection.[152] β-haemolytic group G streptococci are part of the normal microflora of the skin, pharynx, gastrointestinal tract, and vagina. They are important opportunistic pathogens, causing serious infections. The most common portal of entry is the skin, and pharyngeal colonization is common, but foodborne outbreaks have been reported. Group G streptococcal pharyngitis reported in two foodborne outbreaks resembled group A streptococcal pharyngitis, but without the group A sequelae.[10,149] In one outbreak, an egg salad was incriminated as the vehicle of infection;[115] in the other outbreak, a chicken salad was suspected, and after the outbreak a cook was found to be positive for group G streptococci.[215]

50.5.2 *Enterococcus*

The enterococci have been recognized since Thiercelin in 1889[221] described them as the "entérocoque" to emphasize their intestinal origin; in 1906, Andrewes and Horder[6] used the name *Streptococcus faecalis* for the enterococcus-type organism that was isolated from a patient with endocarditis. The taxonomy of this group of bacteria has been vague. In 1937 the enterococci were described by Sherman[205] as those organisms that grow in broth containing 6.5% NaCl at 10° C to 45° C, grow at pH 9.6, survive heating at 60° C for 30 minutes, and react with Lancefield group D antisera. Many species and strains of the genus *Enterococcus* do not meet all of these criteria. Sherman[205] also suggested that the enterococci could be differentiated on the basis of their hemolytic and proteolytic reactions; however, hemolysis is plasmid-mediated[124] and therefore inappropriate as a characteristic for species differentiation. When the genus *Enterococcus* was separated from *Streptococcus sensu stricto* and *Lactococcus*,[201] it included the Lancefield group D (fecal) streptococci, *E. faecalis* and *E. faecium*, but not *Streptococcus bovis* or *Streptococcus equinus*. The genus *Enterococcus* forms a cluster with the genera *Carnobacterium*, *Tetragenococcus*, and *Vagococcus*, and it is more closely related to *Listeria* than to *Lactococcus* or *Streptococcus*.[38,55]

The main reservoir for enterococci is the intestinal tract of humans and animals, but the organism also resides in the oral cavity, gall bladder, urethra, and vagina. Enterococci, usually *E. fecalis*, are considered the second most important nosocomial pathogen. *E. faecalis* has inherent resistance to several antibiotics, and it has been identified as a causal agent of human endocarditis, bacteremia, urinary tract, intraabdominal, and soft tissue infections and neonatal sepsis,[151] whereas *E. faecium* is only implicated as the causative agent in 20% of clinical cases due to enterococci.[134,174] Since re-establishing the genus in 1984, 9 species have been transferred from the genus *Streptococcus*, and 10 new species have been added.[55] They frequently are associated with polymicrobial infections of the abdomen and pelvis. Their etiologic significance in these infections is not clear. In developing countries, the enterococci have been associated with infant diarrhea.[67] The importance of the enterococci for food and public health microbiologists is related to their enteral habitat, their use as indicators for food safety, and their possible involvement in foodborne illness.[212] Despite this, enterococci (mainly *E. faecium*) have been studied for a probiotic role in disease prevention and for treatment of intestinal disorders of humans[150] and animals,[225] and they are available commercially.

In food microbiology it was considered important to identify "*Streptococcus durans*" to distinguish between group D streptococci of fecal and nonfecal origin. *S. durans* had a volatile taxonomic history ranging from species to varietal status within the group D streptococci. Based on DNA hybridization studies, it was shown that *E. faecium* consisted of two distinct entities,[79,137] and the "durans" group was proposed as *Enterococcus durans*.[40] It presently is listed as a pathogenic *Enterococcus*. It has been isolated as an intestinal inhabitant of humans and poultry, thus discounting the previously held view that the "durans" variety represents a nonfecal population. *E. durans* was shown to consist of two distinct DNA homology groups. Homology group I has been retained as *E. durans*, while homology group II is highly related to *Enterococcus hirae*, which has been characterized among isolates from chicken and hog intestines.[78] *E. hirae* therefore includes strains of human, bovine, avian, and porcine origin,[144] and is primarily associated with chicken crops and hog intestines. Many of the newly described species of *Enterococcus*, such as *E. durans* and *E. flavescens*, are of clinical origin whereas others, especially the pigmented species, such as *E. mundtii* and *E. casseliflavus*, are primarily of plant origin.[158,188] *E. faecalis* and *E. faecium* are distributed widely in the environment and also have been associated with plants.[158] This means that public health and food microbiologists must exercise caution when interpreting the meaning of enterococci in foods.

There are many reports of enterococci in foods and the food environment. Some researchers believe that enterococci can cause food poisoning,[172] but Bryan[24] considered that "if enterococci are pathogenic, the situation is probably analogous to the association of coliform bacteria with pathogenicity," and that they only become enteropathogenic under special conditions. In instances where the enterococci have been associated with foodborne illness, the symptoms were vague and variable, including nausea, sometimes vomiting, colicky pains, and diarrhea.[52,172] Efforts to prove that the enterococci cause foodborne illness have included studies with human volunteers, but ingestion of various substrates containing large numbers of enterococci or their filtrates gave equivocal results.[24] Some positive tests with human volunteers have been reported, especially with a Vienna sausage containing α-hemolytic streptococci and a broth culture of the same organism, but not from the bacteria-free filtrate.[30] Variable results were reported when 6 of 27 volunteers became ill after consuming different strains of *E. faecalis* grown in foods.[182] Some volunteers ingested more than 1×10^{10} organisms without showing any symptoms. In fact, some of those who did not show symptoms ingested larger doses than those who became ill,[182] showing a marked difference in response between individuals.

Enterococci are commonly isolated from foods. They are more difficult to control in foods than many other bacteria. Not only their presence in the mammalian intestine but also their adaptation to food processing environments, their relative heat resistance, and their growth over a wide temperature range make control difficult. If certain strains are shown to be enteropathogenic, the critical control procedures of rapid chilling of foods below 5° C or holding above 60° C, and reheating of refrigerated foods to > 70° C before serving, will have to be even more strongly emphasized.

50.5.3 Food-Associated Lactic Acid Bacteria

Many lactic acid bacteria are involved in spontaneous fermentations that acidify and preserve foods. A well-developed fermentation industry exists for cheese and other dairy products, fermented sausages and vegetables, and sourdough bread. It is estimated that 25% of the world's foods are fermented, many of which contain large numbers of viable organisms at the time of consumption. The lactic acid bacteria involved in food fermentation include species of the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus*. All of these bacteria have occasionally been isolated from human infections. This has culminated in reviews of the lactic acid bacteria in human infections by Aguirre and Collins,[2] Gasser,[87] and Facklam and Elliott.[71] Reviews of lactobacilli,[217] leuconostocs,[105] and pediococci[43,161] in human infections concluded that some of these bacteria should be viewed as "potential pathogens." Aguirre and Collins in 1993 and Gasser in 1994 acknowledged the beneficial role of lactic acid bacteria in food preservation and probiotics, but they cited numerous cases in which lactic acid bacteria were implicated in human disease; in particular they noted the importance of enterococci in clinical infection.[2] Gasser[87] questioned why the lactic acid bacteria were being isolated more frequently from clinical cases, whether some lactic acid bacteria are becoming pathogenic, and whether some species or strains contain virulence factors. He concluded that the more sophisticated techniques for identifying bacteria could influence this; furthermore, that fastidious bacteria such as lactic acid bacteria may have been overlooked in the past. There is no evidence that the lactic acid bacteria are becoming pathogenic for healthy adults.

In the case of the lactococci, it is possible that they were misidentified as enterococci because these bacteria are physiologically similar. The lactococci were first implicated in human endocarditis in 1985.[237] Most lactococcal infections are caused by *Lactococcus lactis* subspecies *lactis* and *Lactococcus garvieae.*[64,237] Other lactic acid bacteria involved in cases of endocarditis, apart from the enterococci, are *Lactobacillus rhamnosus* (previously *Lactobacillus casei* subspecies *rhamnosus*), *Lactobacillus plantarum*, and *Lactobacillus casei* or *paracasei.* Nonetheless, in surveys of bacteria implicated in infective endocarditis, the incidence of the lactic acid bacteria is extremely low.[87] The principal lactic acid bacteria associated with primary infections of the blood stream are leuconostocs, but the incidence is also rare; *Lactobacillus* infections of the blood stream generally are secondary, resulting from infection with the same organism at another site in the body.[87] Members of the genus *Pediococcus*, primarily *P. acidilactici*, have been implicated in bacteremia and stomach and hepatic abscesses.[71,161] In most cases of infection with lactic acid bacteria, there was a breakdown in the normal host defenses, associated with conditions such as extremes of age, underlying disease, immunosuppressive therapy, and, in particular, after treatment with vancomycin. Leuconostocs and pediococci are vancomycin-resistant. Up to 30% of leuconostocs react with the group D antiserum, and in the past these infections probably were reported as unidentified enterococci. Despite the widespread distribution of leuconostocs and pediococci in foods, they cause relatively few infections; it is assumed that they have very little virulence for healthy humans.[71]

The newer genera of lactic acid bacteria, *Carnobacterium*, *Oenococcus*, *Tetragenococcus*, and *Weissella* have not been associated with human infections; however, this may simply be due to the relatively short time that these organisms have been known. *Leuconostoc paramesenteroides* (now *Weissella mesenteroides*) has been associated with a few cases of human infection.[71] The carnobacteria were previously described as atypical lactobacilli, but since they have been separated as a new genus, it has been shown that they are more closely associated with enterococci than the lactobacilli.[213] The genus *Tetragenococcus* was derived from *Pediococcus halotolerans.* It is important in the fermentation of soy sauce. It has not been associated with human infections.

50.5.4 Aerococci and Other Lactic Acid Bacteria

The genus *Aerococcus* forms tetrads and contains the species *A. viridans*, known by the synonyms *Gaffkya homari* or *Pediococcus homari*, which causes a fatal disease of lobster.[116] *A. viridans* has been associated also with endocarditis with or without predisposing disease conditions, bacteremia in patients with leukemia, meningitis in children, osteomyelitis, and septic arthritis.[1] *Aerococcus urinae* is an opportunistic pathogen of the urinary tract, primarily among the elderly, especially those with predisposing conditions of diabetes or cancer.[34] The aerococci are widely distributed in nature, in air, water, and soil, hospital environments, plant material, and meat-curing brines;[1] the possible involvement of aerococci in foodborne disease is discussed in Chapter 38.

There are several new genera that have been isolated from clinical sources and that fit the description of lactic acid bacteria; however, their ecological distribution generally is not known. *Gemella* are Gram-variable diplococci and comprise two species: *G. haemolysans* (previously *Neisseria haemolysans*) and *G. morbillorum* (previously *Peptostreptococcus morbillorum*). They are commensals of the mucous membranes of humans and some warm-blooded animals.[16] *G. haemolysans* is commonly found in the intestinal tract of humans. These bacteria are opportunistic pathogens, primarily of immunocompromised individuals. The genus *Globicatella* is closely related to the aerococci, but they form short chains not tetrads. They have been isolated from cases of bacteremia and urinary tract infections. They are a genetically distinct group with closest affinity to the *Aerococcus* phylogenetic branch of the lactic acid bacteria. The closely related genus *Helcococcus* has been isolated from wound infections[39] and *Alloiococcus* from middle ear infections.[71] The genus *Vagococcus* was proposed for the motile lactococci. They are more closely related to the enterococci than to the lactococci. There are two species, *V. fluvialis* and *V. salmoninarum*, but they are rarely associated with clinical infections.[71]

50.5.5 Bifidobacteria

The genus *Bifidobacterium* is no longer considered as one of the lactic acid bacteria because of their relatively high G+C content,[210] but the genus originated from bacteria that were originally classified as *Lactobacillus bifidum.* The bifidobacteria were originally isolated from infant feces and they are currently attracting considerable interest for their role as probiotics. Bifidobacteria have been isolated from polymicrobial infections of children, often with predisposing conditions.[22]

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50.6 HUMAN DISEASES (AND RELATED BACTERIA) TRANSMITTED THROUGH FOODS

50.6.1 Coryneform Bacteria

The coryneform bacteria are included in the actinomycete branch of gram-positive bacteria because of the high G+C content of their DNA. They comprise a group of aerobically growing, nonsporeforming, irregularly shaped, Gram-positive bacteria. There are many medically relevant organisms, including the primary human pathogen, *Corynebacterium diphtheriae.*[84] In addition to the well-defined diphtheria organisms, many nondiphtheria corynebacteria exist, some of which have a pathogenic role. The coryneform bacteria have been associated with an increasing number of cases of disease. They are among the most ubiquitous organisms in the environment, being widely distributed in fresh and salt water, soil, and air. Nondiphtheria corynebacteria constitute a major component of the bacterial flora of human skin. The *C. diphtheriae* group includes the four biotypes of *C. diphtheriae* as well as *C. pseudotuberculosis* and *C. ulcerans.*[84] Not all *C. diphtheriae* are toxinogenic, but all of these species can harbor the phage-mediated diphtheria toxin gene, βtox. The classic symptom of diphtheria is a pharyngeal lesion consisting of a tightly adhering pseudomembrane. The exotoxin is produced by the organism in the local lesion, and it is distributed throughout the body in the blood. The toxin affects the heart and peripheral nerves.

The primary source of *C. diphtheriae* is the nasopharynx of a clinical case, convalescent carriers, or foods. Human skin is the main reservoir of *C. diphtheriae* and transmission from the skin is more effective than from the throat.[84] In both tropical and temperate climates, cutaneous diphtheria is a potential source of *C. diphtheriae* infection.[44] It has been implicated in bovine mastitis and it has been isolated from udder and teat lesions. Milkborne outbreaks of diphtheria generally predate widespread immunization of children and pasteurization of milk.[24] In 1981, a possible foodborne outbreak of diphtheria occurred in the Yemen, in which one of the suspected foods was yogurt.[130] Pasteurization or boiling of milk is an essential precaution for control of corynebacteria. Toxinogenic *C. ulcerans* can cause diphtheria and it may also produce a second toxin that is unaffected by the *C. diphtheriae* antitoxin, but resembles the toxin of *C. pseudotuberculosis.*[44] *C. ulcerans* can cause mastitis in cows and has been isolated from raw milk.[109,235] In the case of an office worker who developed a sore throat caused by a toxinogenic strain of *C. ulcerans*, the source of the organism was traced to raw milk, a farm worker who was an asymptomatic carrier of *C. ulcerans*, and the organism was isolated from two cows.[20] This provides further evidence that *C. ulcerans* is a zoonosis, and adds to the justification for pasteurization of milk for improved public health.

In 1982, Lipsky et al.[153] reviewed infections caused by nondiphtheria corynebacteria. Several species are involved in human infections either as zoonoses from occupational exposure to infected animals, for example, *Corynebacterium equi* and *C. pseudotuberculosis (C. ovis)*, or as opportunistic pathogens, for example, *Corynebacterium bovis*, *Corynebacterium haemolyticum*, and *Corynebacterium xerosis. C. bovis* is a common commensal of the bovine udder that can cause mastitis, but there is no indication that human infections occur through consumption of contaminated milk.[153] Several organisms, previously considered to be pathogenic corynebacteria, have been transferred to other genera, for example, *Corynebacterium pyogenes* has been transferred to *Actinomyces*, and *C. haemolyticum* is reclassified as *Arcanobacterium haemolyticum.*[44] Both of these organisms are opportunistic human pathogens, but they have not been associated with foodborne disease. *Corynebacterium pseudodiphtheriticum (C. hofmannii)* occurs as a commensal of the nasopharynx of humans, but the organism does not produce toxins. It is an opportunistic pathogen causing endocarditis and urinary tract infections.[44] Nontoxinogenic strains are commonly found as part of the normal flora of the upper respiratory tract.[183] There are other coryneform bacteria belonging to the genera *Brevibacterium* and *Propionibacterium* that have been implicated as opportunistic pathogens; but those species associated with foods, *B. linens* in surface-ripened, smear-type cheeses, and *P. shermanii* in Swiss-style cheeses have not been cited among opportunistic pathogens of these genera.[84]

50.6.2 *Mycobacteriaceae*

The mycobacteria are a large heterogeneous group of acid- and alcohol-fast, nonsporeforming bacilli that are found in soil, water, food, and animals. The pathogenic "tubercle bacilli" are the best known and

Mycobacterium tuberculosis, the cause of traditional human tuberculosis, was chosen as the type species for the genus. Nontuberculous mycobacteria (NTM) or mycobacteria other than tuberculosis (MOTT) became known as "atypical mycobacteria" but they are in fact "typical" mycobacteria.[125] The pathogenic mycobacteria include *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, and *Mycobacterium microti*.[95] *M. bovis* was the primary organism of foodborne tuberculosis.[36] Cow's milk was incriminated as a source of tuberculosis in 1846. Three types of "tubercle bacilli" (human, bovine, and avian), as well as many saprophytic mycobacteria, have been identified. Bovine strains caused pulmonary and extrapulmonary tuberculosis in humans;[36] whereas, the avian strain *Mycobacterium avium* was considered to cause tuberculosis of poultry and swine but not humans. The incidence of bovine tuberculosis decreased dramatically in developed countries because of active control programs for dairy herds and other livestock; however, the host range on *M. bovis* is great and includes animal species from badgers to seals.[95] Despite the marked decrease in tuberculosis in developed countries, it remains a major concern in world health.[48] The HIV pandemic has drawn attention to this, but most cases of tuberculosis are not HIV-related.[48,95] It is generally considered that only a few organisms are necessary to cause a respiratory infection; however, except in children, several million organisms appear to be necessary to cause infection by the intestinal route.[82]

Since the 1950s, there has been an increasing reported frequency of NTM disease. This has been attributed to both an increased number of immunocompromised patients and improved technical capabilities of clinical laboratories. The *Mycobacterium avium* complex (MAC) consists of *M. avium* and *Mycobacterium intracellulare*. Originally MAC included *Mycobacterium scrofulaceum*, but this is now considered to be a distinct species.[73] *M. avium* causes human disease, especially in AIDS patients. The *M. avium-M. intracellulare* organisms cause human disease worldwide, especially in northern temperate countries such as Canada, Europe, Japan, and the United States (US).[125] MAC are ubiquitous, and they have been isolated from fresh and salt water, soil, plants, bedding material, and house dust.[125] *M. avium* is responsible for most of the tuberculous lesions found in poultry and swine.[125] Pork and poultry meat could be a source of these organisms, especially if they are not sufficiently cooked. *M. avium* commonly is excreted in feces of birds but not swine, and the organisms can persist in the soil for long periods of time. Direct transmission of *M. avium* from animals to humans is considered to be rare. The primary route of infection in AIDS patients is by colonization of the gastrointestinal tract with large numbers of mycobacteria that infiltrate the intestinal mucosa and subsequently cause bacteremia.[46] Gastric achlorhydria and oral antibiotics are suspected in *M. avium* infections.[125] The thermal death time of *M. bovis* in milk is such that standard pasteurization treatments are expected to destroy the organism with a considerable margin of safety.[135] At 60° C, *M. bovis* dies rapidly in meats.[166] *M. avium-M. intracellulare* organisms are more heat-resistant than *M. bovis*, and they require heating to 71.1° C for 2 minutes to achieve a 12D kill in meat.[166,167]

M. scrofulaceum has been associated with infected lymph nodes in children. It has been isolated from raw milk and other dairy products,[31,162] raw oysters,[120] and water.[73] *Mycobacterium fortuitum* is one of a group of rapidly growing mycobacteria that cause opportunistic focal and disseminated human infections and have been isolated from water, soil, and raw milk.[62] *Mycobacterium gordonae*, previously known as "tap water bacillus" or *Mycobacterium aquae*, has been recovered also from raw milk[120] and can multiply in soil.[218] *M. gordonae* is considered to be nonpathogenic, except under unusual circumstances.[236] Prior to the AIDS epidemic, NTM infections due to *Mycobacterium kansasii* were relatively important, but its importance has been eclipsed by MAC infections. Its reservoir is unknown, but it is occasionally cultured from nonhuman sources, such as water.[236] A major change in awareness of human mycobacterial diseases has come about with the HIV pandemic.

Person-to-person transmission of NTM is rare; therefore, infections are probably derived from the natural environment, where the organisms abound. Organisms of the MAC complex have occasionally been associated with diseases of animals, and they have been isolated from raw milk, cheese, ice cream, and butter,[31] but the principal source of NTM seems to be water.[73] There are many questions to be answered about the changing epidemiology of NTM in both immunocompetent and immunosuppressed individuals. In immunosuppressed patients it appears that colonization of the intestine is a major step in the pathogenesis of the disease, making water and food the most likely sources of infection. The mycobacteria generally are hardy organisms that survive for long periods of time in the environment and they can be difficult to eliminate with disinfecting chemicals.

A role for mycobacteria in the etiology of Crohn's disease—an inflammatory bowel disease analogous to infectious ileitis in ruminants, known as Johne's disease—has long been the subject of speculation. Johne's and Crohn's diseases have been associated with *M. paratuberculosis* and *M. avium* A/I (the predominant strain causing tuberculosis of animals and birds).[165] *M. paratuberculosis* is always assumed to be pathogenic.[165] Crohn's disease is most likely a multifactorial disease involving genetic, environmental, and infectious elements. The association of these organisms with animal tuberculosis compels the question of food as a source of infections in humans, if the same organisms can be confirmed as causative agents in Crohn's disease. For further information, see reviews by Chiodini[32] and McFadden and Fidler.[165]

50.7 ZOONOSES TRANSMITTED THROUGH FOODS

Zoonoses are infectious diseases that can be contracted by humans in which the pathogen is normally associated with an animal population. They are rare in developed countries, but they still ravage the populations of developing countries. More than 150 zoonoses have been described, some of which can be foodborne, such as anthrax, bovine tuberculosis, brucellosis, Q fever, and some *Salmonella* infections. Zoonoses are not limited to bacteria; many parasites and some viruses are also involved.

50.7.1 Anthrax

Herbivores are highly susceptible to anthrax, whereas swine and humans are relatively resistant.[206] Immunization has decreased its incidence among farm animals, but the disease remains prevalent in many countries, and cases of human anthrax are still reported.[206] The outbreak of anthrax in Zimbabwe between 1978 and 1980 emphasized the need for ongoing animal health programs.[50] Human anthrax is classified as industrial or nonindustrial. Industrial anthrax is an occupational hazard of working in industries that process animal products, and can occur by inhalation (wool-sorters' disease). The most common method of spread of this disease is through diseased animals and carrion contaminating the soil. Human anthrax most commonly is contracted cutaneously through cuts and abrasions and rarely by ingestion.[224] Incidents of intestinal anthrax almost invariably have involved the ingestion of raw or poorly cooked meat from infected animals. Because of the severity of the disease in animals, antemortem and postmortem inspection should eliminate infected animals from the food supply. The obvious precaution of rejecting diseased and dead animals for human consumption aids in the control of intestinal anthrax. Adequate cooking of meats protects consumers from exposure to vegetative cells of *B. anthracis.* Intestinal anthrax from consumption of milk is rare because the animals are usually very sick and their milk visibly is abnormal.[24] Cutaneous infections form a characteristic pimple-like projection that forms a black eschar (similar to a scab). Intestinal anthrax develops in the same manner as cutaneous anthrax, by forming a necrotic lesion. The incubation period is usually 2 to 5 days, at which time a high temperature, general weakness, gastroenteritis (nausea, vomiting, and diarrhea), abdominal pain, and prostration may occur. The disease may progress to a generalized toxemia associated with 25% to 50% mortality.[224] Lack of motility is a major criterion for differentiating *B. anthracis* from all other *Bacillus* spp. Virulent *B. anthracis* grown at elevated CO_2 concentrations form mucoid colonies due to cell encapsulation.[60] *B. anthracis* produces a "medusa head" appearance on aging colonies on laboratory media, which often is cited as a differentiating characteristic. However, other *Bacillus* also produce this feature, including *B. cereus*, *B. thuringiensis*, and *B. cereus* var. *mycoides.*[184]

50.7.2 Brucellosis

Despite advances in the control of animal brucellosis, it remains a worldwide health concern for animals and humans. Most cases of human brucellosis can be traced to contact with animals and meat handling at abattoirs,[26–28] but a significant number of cases are contracted through unpasteurized milk and milk products. This is ongoing in Mediterranean and Middle Eastern countries, Pakistan, and Tanzania,[41] and in California an increasing incidence of foodborne transmission has been reported among people of Hispanic origin.[33] Cases of brucellosis frequently are unrecognized, and mild cases generally are underreported. The brucellae are classic organisms in bacteriology. They were recognized before the twentieth century for their causal role in Malta (undulant) fever, an incapacitating disease of humans and, separately, as the causal agent of the economically devastating Bang's disease (contagious abortion) of cattle. There are three "classic" species in the genus *Brucella*: *B. melitensis*, *B. suis*, and *B. abortus*. *B. melitensis* is a primary pathogen for goats and sheep, *B. suis* for swine, and *B. abortus* for cattle, but host specificity has declined.[173] Brucellae cause diseases of many other domesticated and semidomesticated animals, and all three species cause undulant fever in humans.[173] *B. melitensis* is the most pathogenic for humans, followed by *B. suis* and *B. abortus*. Hence, goat-raising areas such as the Mediterranean littoral and Mexico are more likely to be areas of high incidence of brucellosis due to *B. melitensis* than the US, Canada, or central Europe. In the latter areas, *B. suis* or *B. abortus* predominate.[191] The other three species, *Brucella neotomae*, *Brucella canis*, and *Brucella ovis* are referred to as the "new" species.[173] *B. canis* is a primary pathogen of dogs. It has been associated with "milder forms" of human brucellosis.[219] Brucellae isolated from sea mammals have been tentatively named *Brucella maris*.[194]

Brucellae generally grow only in the host; however, they survive well outside of the host.[173] In vivo these organisms produce L-forms, which makes them nonculturable; this is a factor contributing to their chronicity and diversity.[111] They are nonmotile, nonsporulating coccobacilli or short rods. The organisms are strict aerobes, but some grow best in an atmosphere with 5% to 10% CO_2.[173] Brucellae have fastidious growth requirements, including biotin, thiamine, nicotinamide, and traces of magnesium. They can be difficult to propagate on ordinary culture media,[42,111] some require the addition of serum, and growth is usually better in liquid rather than on solid media.[173]

Human brucellosis is primarily an occupational disease of veterinarians and meat workers.[26–28] Brucellae can be spread by aerosols and dust.[133] Foodborne cases of undulant fever occur often as a result of ingesting unpasteurized milk and milk products.[185,186] Undulant fever is a severe disease but it does not have specific signs or symptoms. Even the symptom for which it is named, a rising and falling fever, may not occur. The severity of the disease depends to some extent on the species of *Brucella* involved. The incubation period is usually 3 to 21 days, but it can be as long as 7 months. Onset of symptoms can be gradual or abrupt. In its acute form, the predominant symptoms are pyrexia, profuse sweats, chills, weakness, malaise, body aches, chest and joint pains, weight loss, and anorexia.[26,234] In its chronic form (a state of infection that has lasted more than 1 year, but bacteria usually cannot be isolated), there is a long history of bouts of fever, malaise, headache, sweating, recurrent depression, inertia, vague pains, sexual impotence, and insomnia.[234] The disease can be incapacitating, leading to permanent invalidism.[173] Complications occur in 10% to 15% of patients; osteomyelitis is the most common complication of *B. melitensis* infections. Other complications include skeletal, genitourinary, cardiovascular, and neurological conditions.

In animals, the organisms localize in the uterus of pregnant animals and in the mammary glands of lactating females.[173] After the abortive stage, infected animals can shed brucellae intermittently in varying numbers in their milk for years.[173] Consumption of unpasteurized goat's milk cheese has been implicated in many incidents of undulant fever,[8,68,242] as has unpasteurized sheep's milk cheese.[86] Brucellae remain viable in goat's milk cheese for up to 100 days. They survive up to 6 months but not as long as 1 year in Cheddar cheese stored at 4.4° C.[91] They also survive for long periods in raw meats, but not in smoked (heated) products.[123] Meats are rarely implicated in outbreaks of undulant fever, because cooking usually is sufficient to destroy the *Brucella* organisms. Bacteriological confirmation of infection occurs in less than 20% of patients with high antibody titres. Isolation of brucellae from clinical specimens is irrefutable evidence of infection; however, diagnosis usually is based on serological results.[173] Serological detection in chronic infections can be a problem because levels of antibody titer vary with stages of the disease.[163] Instances of elevated antibody titers without infection have been recorded with animal workers. The organisms are highly invasive; however, they do not usually cause a bacteremia because they are facultative intracellular parasites.[173] This makes the disease difficult to treat with antibiotics, so prolonged antibiotic treatment is advised.[173]

Some countries have managed to eradicate brucellosis, but this is the exception rather than the rule.[185] In the US and Canada, programs designed to decrease the incidence of brucellosis have had a marked effect. In contrast, Mexico still has a high incidence of the disease. Although brucellae are resistant to environmental stress, they are killed rapidly by high temperatures, such as those used in pasteurization and for cooking (smoking) processed meats.[123] Both low-temperature holding and high-temperature, short-time methods of pasteurization of milk are able to eliminate heavy contamination with *B. abor-*

tus.[49] In the case of raw milk cheese, it appears that aging for 90 days is not sufficient to eliminate brucellae. Goat's milk is often recommended as an alternative for infants who are allergic to cow's milk. While goat's milk can be produced even more hygienically than cow's milk, the systems for handling and marketing goat's milk are not as well established and high bacterial counts have been recorded.[169,193] As with cow's milk, it would be prudent to pasteurize goat's milk before consumption. Travelers to areas where brucellosis is endemic, especially goat-raising areas, should be cautious about consuming cheeses and other dairy products prepared from unpasteurized milk.

50.7.3 Q Fever

Q (Query) fever in humans is caused by *Coxiella burnetii*, a minute, filterable, pleomorphic cell that is generally Gram-negative.[93] *C. burnetii* originally was named *Rickettsia burneti* because it is a rickettsia-like organism. It is an obligate intracellular parasite that localizes and proliferates in cell vacuoles, as opposed to *Rickettsia* species, which grow within the cell cytoplasm. *C. burnetii* is also differentiated from typical rickettsiae by its G+C content of 42% compared with 29% to 33% for other rickettsiae.[190] It is remarkably resistant to physical and chemical stress. The organism generally is regarded as a nonspore former; however, spore-like formations were observed in the periplasmic space of large cell variants.[164] "Spore" formation could account for the exceptional resistance of *C. burnetii* to adverse conditions.

Q fever was first described among meat plant workers in Brisbane, Australia, in 1935.[53] Since then, it has become recognized worldwide as a disease of humans. Q fever is enzootic but generally asymptomatic in cattle, sheep, goats, and other animals. The illness is usually contracted by humans as an occupational disease. Typical features of Q fever in humans include an initial fever, followed by malaise, anorexia, muscular pain, and intense headache. Misdiagnosis is possible because the symptoms resemble viral influenza. During the febrile stage, bacteremia occurs and the organism can be recovered from urine. A gradual abatement of fever occurs after 1 to 2 weeks, and the organism disappears from the blood. The usual incubation period is 2 to 4 weeks, varying with the route of exposure, the infective dose, and the age of the individual.[54] Complicating sequelae include coughing, chest pain, liver damage, and endocarditis in latent or chronic cases.[190] The incidence of Q fever is probably much greater than reported.

The most common route of transmission from animals to humans is by aerosols, especially from parturient dairy cows, sheep, goats, and other animals, including cats and dogs. The principal modes of infection include inhalation of contaminated dust at meat plants and dairies, handling and ingestion of contaminated meat or milk, and handling of infected carcasses.[12] Most evidence of transmission of *C. burnetii* by milk is circumstantial. A common source outbreak from raw milk has been reported;[23] however, in a study in which contaminated raw milk was ingested by human volunteers, infection did not occur.[148] In milk, *C. burnetii* is more resistant to heat treatment than *M. tuberculosis*. Heating milk at 62.8° C for 30 minutes and 71.1° C for 15 seconds is sufficient to kill *C. burnetii*. These time-temperature standards have been adopted for pasteurization to prevent milkborne Q fever.[66]

50.7.4 Leptospirosis

This disease is thought to be the most widespread zoonosis in the world. The causative agent, *Leptospira* spp., has a natural reservoir in wild animals, particularly rodents, and domesticated animals, including cattle and dogs.[227] *Leptospira* are aerobic spirochetes that are motile, containing two periplasmic flagella inserted subterminally at opposite ends of the cell.[4] *Leptospira interrogans* is a pathogen that causes acute, febrile, systemic disease of humans and other mammals. Pathogenic and saprophytic strains are morphologically and biochemically indistinguishable. The rat *(Rattus norvegicus)* is the principal carrier of *L. interrogans* var. *icterohaemorrhagiae.*[102] Humans are an accidental host of leptospiral infections. The disease is primarily an occupational risk for veterinarians, dairy and hog farmers, and meat, poultry, and fish processors.[4,227] Leptospiraemia at the time of onset of disease persists for approximately 1 week. During the acute phase, leptospires can be detected in body fluids, including milk. Thereafter, the organisms become localized in the kidney tubules, whence they contaminate the urine of infected animals.[4] Cattle-associated leptospirosis caused by serovar *hardjo* of *L. interrogans* var. *hebdomadis*[17,227] causes a milder form of leptospirosis in humans that is a flu-like illness with symptoms including fever and headache, in which mental confusion often occurs. Milk from cows with acute leptospirosis could be contaminated with *Leptospira* spp. The incidence of foodborne leptospirosis is limited because of a leptosporicidal effect in raw milk that causes viable cells to disappear within 3 hours.[139]

50.7.5 Erysipelas

Erysipelothrix rhusiopathiae, also known as *Erysipelothrix insidiosa,* is a microaerophilic, nonmotile, Gram-positive rod belonging to the family *Corynebacteriaceae.*[230] The Gram-stain decolorizes rapidly, so it may appear to be Gram-negative.[98] *E. rhusiopathiae* is widely distributed in nature, usually associated with decaying organic matter. It is generally ubiquitous to the environment of swine.[238,239] *E. rhusiopathiae* is pathogenic for swine,[238] but humans are relatively immune to infection, except as an occupational disease through contact with infected animals or by handling contaminated animal products, including pork, poultry, and fish.[98,230] The most common form of human infection is a mild, localized inflammatory, nonsuppurative lesion (erysipeloid) on the fingers or hands.[98,230] This form of the disease is self-limiting. A severe, generalized cutaneous form, and sepsis with or without cutaneous involvement can occur, with endocarditis as a possible but rare sequela.[98,230] *E. rhusiopathiae* infection caused by ingestion is rare; however, the organism survives salting, pickling, and smoking treatments,[98] and it survives in such meats for 1 to 3 months.[24] The organism commonly can be isolated from work tables and pork in meat plants.

50.7.6 Tularaemia

Francisella tularensis, the causative agent of tularaemia, is a small, aerobic, nonmotile, faintly staining Gram-negative pleomorphic coccobacillus or rod, which may possess a single flagellum.[231] There are many ways in which this organism is transmitted to humans. The principal mode of transmission is from blood-sucking arthropods, especially ticks, which serve not only as the main vector but also as the main reservoir of the organism.[199] Ingestion of insufficiently cooked, infected wild rabbit meat, contaminated water, or rodent-contaminated foods has been implicated in infection.[118] The clinical symptoms associated with the disease depend on the mode of transmission. The typhoidal form is associated with ingestion of contaminated meat or water. The symptoms resemble typhoid, including fever, prostration, nausea, vomiting, abdominal pain, and diarrhea. Ulcerative lesions of the buccal mucosa, pharynx, and intestine can occur, and the cervical, pharyngeal, and mesenteric lymph nodes can become involved.[69] The organism is extremely virulent. As few as 10 to 50 organisms cause infection subcutaneously or by the respiratory route; however, doses as high as 10^{10} organisms are considered necessary for infection by the gastrointestinal route.[24,69] The disease is limited to the northern hemisphere, where it has been associated with approximately 100 species of wild mammals, 9 species of domesticated animals, 25 species of birds, and several species of fish.[118] Despite this, animals are not considered to be a real reservoir of these organisms. Domesticated rabbits are susceptible to tularaemia, but they are rarely infected.[24] *F. tularensis* retains its virulence for long periods during frozen storage in rabbit meat, but the organism is not heat-resistant and can be killed by exposure to 56° C to 58° C,[24] making its survival in appropriately cooked meat unlikely.

50.7.7 Intestinal Spirochetosis

Spirochetes have long been known to be present in the human intestinal tract, but their clinical significance is uncertain. Some investigators have reported that massive infestation of spirochetes in the colonic epithelium may cause diarrhea.[58,85,108] In general, very little is known about intestinal spirochetes, except for *Treponema hyodysenteriae,* which was recognized as a cause of swine dysentery in the early 1970s.[107,220] Swine dysentery is a mucohemorrhagic diarrhea, resulting from inflammation of the colon, with excess mucus production and superficial necrosis that causes hemorrhage, dehydration, and possible death.[138,220] *T. hyodysenteriae* is a large spirochete.[108] It is an anaerobic, Gram-negative, oxygen-tolerant, helical coiled organism, containing one or more periplasmic flagella at each end of the cell.[207] Treponemes are not considered to be normally part of the human intestinal microflora;[207] however, Harris et al.[108] have reported that treponemes are present in the intestinal flora of most animals, including humans. A new spirochete, *Brachyspira aalborgi,* was described as a parasite of humans.[121] The treponemes are host-associated organisms, and *T. hyodysenteriae* causing swine dysentery might not be pathogenic for humans. Anaerobic spirochetes are difficult to grow in the laboratory; however, media containing 4 mg spectinomycin ml^{-1} will allow selective growth of *T. hyodysenteriae.*[209] At this time, *T. hyodysenteriae* is not implicated as a human pathogen, but it will be interesting to observe the future developments in our knowledge of this organism and other intestinal spirochetes.

50.8 CONCLUDING REMARKS

The challenge of writing a review on the less recognized and suspected foodborne bacterial pathogens has changed dramatically with the acceptance by clinical microbiologists that production of disease does not reside solely with the pathogenic and virulence properties of the microorganisms but also with the health status of the host.[126] This brings into question the safety of refrigerated and fermented foods, as well as many raw vegetables, in which large populations of viable bacteria could be present at the time of consumption. It is clear that the general spoilage and fermentation bacteria do not have established virulence characteristics, but that they can be opportunistic pathogens. The best illustration of this is the fact that all of the lactic acid bacteria involved in lactic fermentation of foods and bifidobacteria that are used as probiotic cultures have been implicated in cases of human infection; however, individuals who become infected with these bacteria almost invariably have an underlying disease and, in the case of leuconostoc and pediococcus infections, they have usually been treated with vancomycin for another infection. Both leuconostoc and pediococci are vancomycin-resistant.[71] Consumer demand for minimally processed foods with a long refrigerated storage life, which can be achieved with vacuum or modified atmosphere packaged foods with elevated levels of carbon dioxide, provides a niche for the growth of lactic acid bacteria. This includes fermented dairy products and fermented meats, such as chill-stored vacuum-packaged, sliced processed meats. It is prudent for immunocompromised individuals to avoid such foods in their diet unless they are heated to internal temperature of 70° C before consumption. Despite this, it is reasonable to conclude that these foods represent an extremely low level of risk for the population at large. Unfortunately, for susceptible individuals, even the irradiation of foods would not solve the problem because the most likely surviving organisms are lactic acid bacteria.[110]

In contrast, the enterococci represent a much more serious dilemma for food safety. It was suggested by Bryan[24] that specific strains of enterococci may have virulence traits. This has not been demonstrated to date, but it is clear that the enterococci are more likely to become established as potential pathogens than the closely related lactic acid bacteria. The enterococci are commonly associated with foods as fecal contaminants, but their resistance to the extraenteral environment, heat, and sanitizing chemicals makes them a natural microorganism to survive in a food processing environment. *E. faecalis* is the principal species involved in human infections, but infections due to vancomycin-resistant strains (VRE) of *E. faecium* are causing concern.[97] The safety of enterococci is a challenge for food technology and public health. While enterococci are recognized as a major concern in nosocomial infections, especially with VRE, the enterococci are considered as an important component of some food fermentations and they are used by some people as probiotics. Clearly, clarification of these anomalies is required.

The Enterobacteriaceae represent an interesting source of potential or opportunistic pathogens in the food supply. Enterotoxigenic strains of both *C. freundii* and *K. pneumoniae* can be considered foodborne pathogens in the same way that specific strains of *E. coli* have become recognized foodborne pathogens. Until the outbreak of hemorrhagic colitis and HUS from *C. freundii* in parsley butter in 1995,[223] it was assumed that the most likely type of enteropathogenicity in other Enterobacteriaceae would be cholera-like enterotoxigenicity, not the shiga-like enterocolitis. The possibility that these strains also have a low infective dose represents an added challenge for food safety. In general, the Enterobacteriaceae do not grow at refrigeration temperatures, but even in the case of *Yersinia enterocolitic*, which is psychrotrophic, refrigerated foods have not been implicated in foodborne illness. Other species of Enterobacteriaceae are psychrotrophic, including *Pantoea agglomerans* and *Serratia liquefaciens*, but their role in food spoilage or as potential enteropathogens is not known. Strains of other Enterobacteriaceae, such as *H. alvei*, seem more likely to become a concern in food safety than the psychrotrophic species.

Ensuring the microbial safety of foods in the future will present many challenges for food technologists and public health authorities.

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51 Protozoa

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51.1 INTRODUCTION

Protozoa are micro-organisms with either simple or complex structures contained within a single cell, and equally simple or intricate life cycles that vary considerably depending on the species. Since their discovery, some 65,000 species have been described.[153] Protozoan parasites are responsible for some of the most globally important diseases of man and animals throughout the world. Malaria, for example, affects 280 million people annually, of which 110 million develop clinical disease each year.[143] Protozoan parasites often have complex life cycles with transmission occurring between two or more species of host either via biting or blood-feeding arthropod vectors, or by predation involving ingestion of tissue stages present in infected meat. Many of the protozoa with a more simple and direct life cycle can encyst, ensuring survival and a means of transmission from one host to another.

Outbreaks of gastrointestinal disease in man associated with foodborne and waterborne microorganisms have received much publicity against a background of an ever-expanding range and prevalence of diseases. In the United Kingdom (UK), there were 4,317 and 5,294 reports of gastrointestinal disease caused by *Cryptosporidium* and *Giardia*, respectively, reported throughout England and Wales in 1997.[54] In many cases, infections due to protozoan parasites are the result of poor hygiene and occur most often among travelers, particularly those visiting underdeveloped countries. However, an increasing number of opportunistic protozoan infections are reported in the elderly or the immunocompromised resident in industrialized countries.

Reports of foodborne transmission of protozoa have been relatively uncommon; however, foodborne disease due to protozoa may be more common than reports suggest, possibly underdetected by a factor of 10 or more.[44] In many foodborne outbreaks, an etiological agent is identified in less than half of cases.[16,52] In contrast, protozoa often are implicated in waterborne gastrointestinal disease with several large-scale disease outbreaks reported throughout the world. It is probably true to say, though, that prevention and control of protozoal disease outbreaks have been hindered by a lack of surveillance procedures and limited availability of appropriate diagnostic tests for protozoan parasites, particularly in food.

Some protozoan parasites involved in foodborne and waterborne disease outbreaks also occur in animals. With some of these infections there is good evidence for zoonotic transmission; however, in many cases conclusive evidence for animal-to-man transmission is lacking.

Of nearly 40 species of protozoa that infect humans, this chapter will describe the life cycles, incidence, symptoms, and methods of detection of some of the more well-recognized, as well as the emerging genera of foodborne and waterborne pathogens. Classification and characteristics of these protozoan parasites are summarized in Table 51–1.

A number of other protozoa have the potential for transmission by food or water. These include the following:

- *Naegleria fowleri* is a free-living, soil-dwelling amoeba that can infect the olfactory and nasal passages and is a rare cause of meningioencephalitis. The organism is transmitted via water following swimming or bathing.[163]
- *Acanthamoeba* are opportunistic pathogens that can produce encephalitis, keratitis, and pneumonitis. Infection is thought to occur via inhalation, but keratitis may be associated with transmission to the eye from domestic tap water, particularly in contact lens wearers.[229]
- *Isospora belli*, a coccidian parasite of man.
- The flagellates—*Chilomastix mesneli*, *Enteromonas hominis*, *Retortamonas intestinalis*, *Trichomonas hominis*, and *Trichomonas tenax*.
- The amoebae—*Dientamoeba fragilis*, *Entamoeba coli*, and *Iodamoeba buetschlii*.[42]

51.2 APICOMPLEXAN (COCCIDIAN) PROTOZOA

51. 2.1 *Cryptosporidium*

History. Protozoa of the coccidial genus *Cryptosporidium* are small intracellular parasites that occur throughout the animal kingdom and have been reported in many species of mammals, birds, reptiles, and fish.[89] Since the discovery of the organism,[258] the importance was overlooked for a long time, until the recognition of infection in various species of domestic animals, especially cattle and sheep, in the 1970s. The taxonomic status of *Cryptosporidium* is still not fully resolved but it is generally accepted that the species that occurs in man and other mammals is *Cryptosporidium parvum*.[57] Other species occurring in mammals are *C. muris* reported in rodents and cattle,[7,258] *C. felis* in cats,[110] and *C. wrairi* in the guinea pig.[50] These latter two species appear to be highly specific to their respective hosts.[110] In birds two species, *C. baileyi* and *C. meleagridis*, have been reported although other species may exist. *C. baileyi* is usually associated with infections of the cloacal bursa, cloaca, and respiratory tract in a number of species of birds.[155] *C. meleagridis* is thought to be the species associated with gut-related infections in turkeys.[109] The avian cryptosporidia appear to have a high host specificity for birds. However, a report of infection with *C. baileyi* in an immunosuppressed human patient[65] suggests that under certain circumstances cross-transmission to mammalian hosts may occur.

Cryptosporidium also are found in lower vertebrates. *C. sepentis* (*C. crotali*) has been reported from the stomach of snakes[27,152] and *C. nasorum* in fish.[126]

Life Cycle. The life cycle of *Cryptosporium parvum* (Figure 51–1A) is similar to that of other coccidian parasites and can be divided into excystation (release of infective sporozoites), merogony or schizogony (asexual multiplication), gametogony (sexual reproduction), and sporogony (sporozoite formation). Each intracellular stage resides within a parasitophorous vacuole confined to the microvillous region of the host cell (Figure 51–1B). The infective stage is the oocyst, which is passed into the environment by infected hosts via the feces. Unlike the situation in many species of coccidian parasite, the oocysts are fully sporulated and immediately infective when excreted, requiring no period of maturation without the host. On ingestion by a new host, sporulated oocysts excyst in the intestines, releasing free sporozoites that penetrate the microvillous border of enterocytes lining the gut epithelium. The sporozoites undergo asexual development or merogony producing mature meronts containing 6 to 8 merozoites (Figure 51–1C). These meronts rupture, releasing the merozoites that infect further host cells producing more meronts. After a period of rapid recycling, a second meront stage is produced with four merozoites, the released merozoites of which develop into microgamonts and macrogamonts. Fusion of a microgamete (male) with a macrogamete (female) produces a zygote that produces an outer wall to form the oocyst. Sporogony occurs within the host and it appears that two types of oocysts are produced; 80% develop a thick, environmentally resistant oocyst wall and are passed in the feces; 20% form thin-walled oocysts that are auto-infective and allow for further recycling.[56]

Prevalence in Man and Animals. Human infection with *C. parvum* has worldwide distribution and has become a common cause of gastrointestinal infection both in immunocompromised individuals, as an opportunistic pathogen associated with acquired immune deficiency syndrome (AIDS), and in otherwise healthy people.[261] In the relatively short time since its discovery in humans,[184] surveys have

Table 51–1 Parasitic Protozoa—Classification Based on Levine

Phylum	*Genera**	*General Characteristics*
Apicomplexa	*Cryptosporidium* *Cyclospora* *Sarcocystis* *Toxoplasma*	Apical complex (visible with electron microscope) generally consisting of polar ring, rhoptries, microneme, conoid; all species parasitic
Sarcomastigophora		
Subphylum Mastigophora	*Giardia*	One or more flagella usually present in trophozoites; asexual reproduction longitudinal
Subphylum Sarcodina	*Entamoeba*	Pseudopoda or locomotive protoplasmic flow without discrete pseudopodia; asexual reproduction by fission
(subphylum Blastocysta†)	*Blastocystis*	Pleomorphic, brightly refractile cysts with a central vacuole. Amoeboid form may occur
Ciliophora	*Balantidium*	Cilated organisms with micronucleus and macronucleus
Microspora	*Encephalitozoon* *Enterocytozoon* *Nosema* *Pleistophora* *Septata*	Unicellular spores with imperforate wall containing a uninucleate or binucleate sporoplasm and extrusion apparatus with polar tube or cap; obligatory intracellular parasite

* Genera of parasitic protozoa referred to in text only

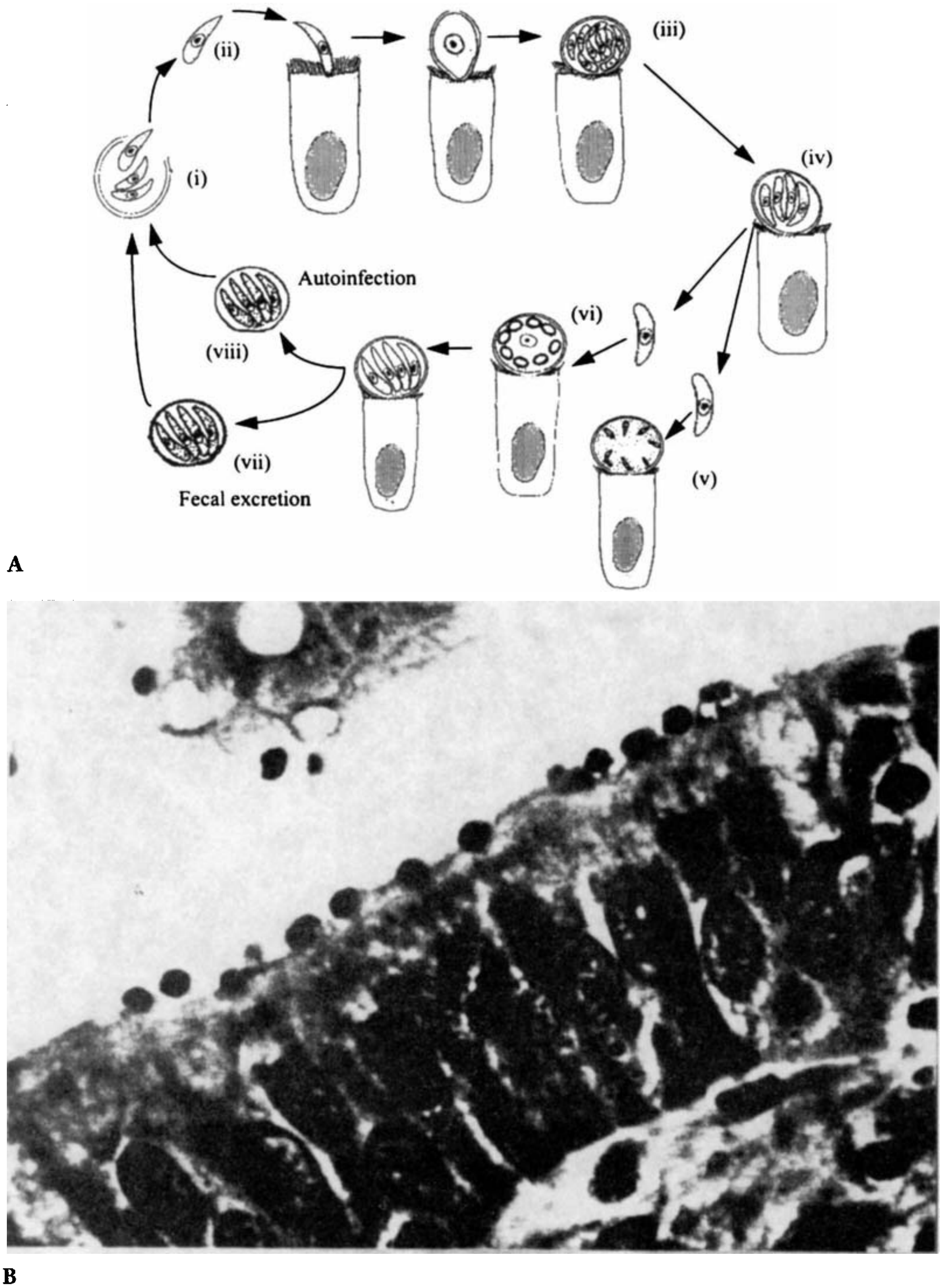
† Jiang and He[133] suggest that *Blastocystis* should be classified in its own subphylum within the phylum sarcomastigophora

Source: Data from N.D. Levine, *Veterinary Protozoology*, © 1985, Iowa State University Press, Ames, Iowa.

shown human infections on all six continents, in both developed and developing countries, and in urban as well as rural locations. In more industrialized countries of Europe and North America, the incidence of clinical infection is generally between 1% and 3% while elsewhere rates of between 4.9% in Asia to 10.4% in Africa have been suggested.[261] The higher prevalence in less developed countries may relate to lack of clean water, sanitation, or general crowding. Seroprevalence studies suggest a higher rate of exposure to cryptosporidiosis with levels between 25% and 35% in Europe and North America, suggesting that many *Cryptosporidium* infections are asymptomatic.[261] Cryptosporidia are found in the feces of 10% to 20% of patients with diarrhea associated with AIDS.[136,147,150,197,213,230,241,282]

Cryptosporidiosis is common in young livestock especially cattle and sheep, although pigs, goats, horses, and deer can be infected. The pathogen infects 79 species of mammals and is highly prevalent in ruminants, particularly in young calves,[89,250] and appears to be age-related. Seasonal peaks of disease have been reported to coincide with birth peaks in spring and autumn.[8] The first calves to be born often become infected without showing clinical symptoms but become sources of infection for calves that follow. Infection spreads rapidly, and later-born calves can become so heavily infected that clinical disease results. Disease associated with *C. parvum* also has been reported in neonates of sheep, goats, and deer and in nondomestic ruminants including antelope and orynx.[112] Horses, goats, and pigs can also be infected. Cryptosporidiosis has been reported in immunodeficient foals[106] as a cause of diarrhea. Most pig cryptosporidial infections are asymptomatic with the majority of infections occurring in 6- to 12-week-old pigs.[154] Clinical signs, where they do occur, include diarrhea and failure to thrive although these usually are associated with the presence of other pathogens such as *Salmonella*, *Escherichia coli*, adenovirus, or *Isospora suis*. There are only a few reports of *Cryptosporidium* infections in companion animals. Dogs, cats, and other pets occasionally are infected but they do not appear to be an important source of infection to other hosts. Given their ubiquitous occurrence, there is a large potential reservoir of infection to man and domestic animals.

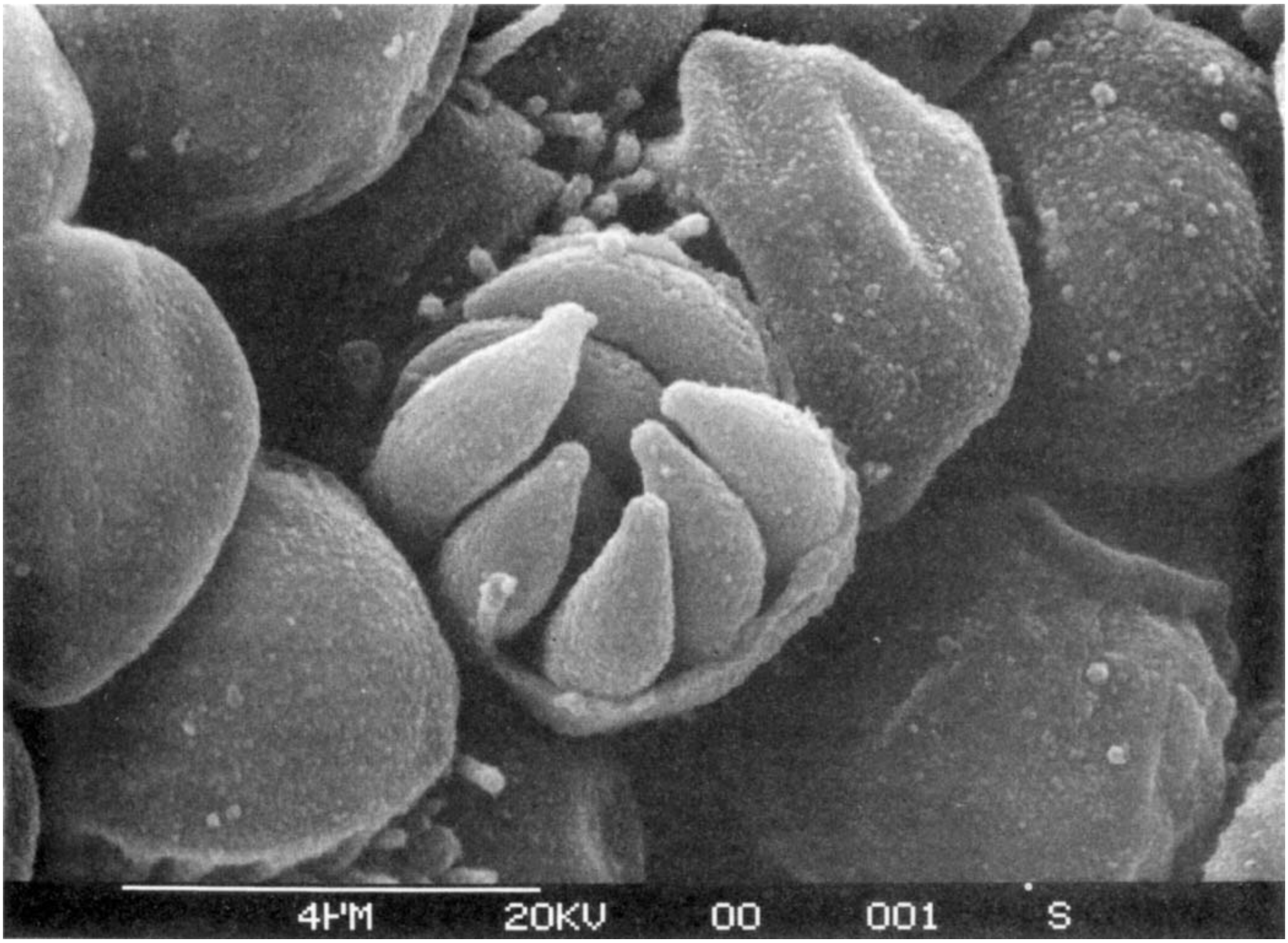
Epidemiology and Transmission. Transmission from host to host is via the oocyst stage by the fecal-oral route through contaminated water, contaminated food, animal-to-person contact, or person-to-person contact. The parasite is highly infectious and seems to be one of the main causes of infectious diarrhea in many parts of the world.[261] Oocysts of *C.*



continues

Figure 51–1 Life Cycle of *Cryptosporidium parvum*. **A**, Sporulated oocysts upon ingestion, excyst (i) in the intestines releasing free sporozoites (ii), which penetrate the microvillous border of gut epithelial cells. The sporozoites undergo asexual reproduction, merogony, to form first generation meronts (iii) containing 6 to 8 merozoites. These rupture releasing merozoites, which form second generation meronts (iv) with 4 merozoites. Released merozoites produce either microgametocytes (v) or macrogametocysts (vi). Fusion produces a zygote that undergoes sporogony within the gut to form either thick-walled oocysts (vii), which pass out in the feces, or thin-walled oocysts (viii) that can autoinfect further epithelial cells within the host. *Source:* Reprinted with permission from M.A. Taylor, Waterborne Parasites: A Veterinary Perspective, *Health and Hygiene*, Vol. 14, p. 56, © 1993, Royal Institute of Public Health and Hygiene. **B**, Trophozoites of *Cryptosporidium* attached to the microvillous border of gut epithelial cells. *Source:* Copyright © 1999, Ministry of Agriculture, Fisheries and Food, U.K.

Figure 51–1 continued



C, Scanning electron micrograph (SEM) of *Cryptosporidium* (× 15,000). The figure shows "banana-shaped" merozoites of first-generation meront of *Cryptosporidium*. *Source:* Copyright © 1999, Ministry of Agriculture, Fisheries and Food, U.K.

parvum, unlike many other coccidia, are fully sporulated when shed and therefore are thought to be infectious immediately.[88] They survive in wet or moist conditions outside the body for weeks or months and survive longer at cold temperatures. Many mammals have been found naturally infected with *C. parvum* and have the potential, therefore, to act as reservoir hosts.

In man, person-to-person transmission is now recognized to be common and is considered to be the main route of transmission.[205,239] Zoonotic transmission has been reported from calves and lambs, particularly following educational visits to farms.[14,240] In Australia, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) has demonstrated geographical differences in strain isolates from different hosts, which suggests local endemnicity and zoonotic transmission.[180] Isoenzyme analysis in the UK has shown differences between human isolates and animal isolates.[168] It is not clear at this stage whether this represents isolate variation in cross-species transmission between Australian or UK isolates or just differences in the strain typing methods. Recent evidence suggests that two distinct strains and transmission cycles of *C. parvum* exist, arbitrarily designated genotype 1 (predominantly of human origin) and genotype 2 (predominantly of animal origin).[12,196]

In many instances where *Cryptosporidium* is diagnosed in animals, it appears that infections usually originate from the same host species. In animals, the primary route of infection is likely to be the direct animal-to-animal fecal-oral route. Thus in calves, for example, overcrowding, stress of early weaning, transport, and marketing, together with low levels of hygiene increase the risk of clinical infections. In lambs, chilling due to adverse weather conditions in the neonatal period, intercurrent infections, or nutritional or mineral deficiencies could exacerbate or increase the likelihood of disease. Infection in these cases is likely to occur through grooming, nuzzling, coprophagy, fecal soiling by direct contact with infected animals, or indirectly through consumption of contaminated feed or environmental sources including pasture and water.[250] Heavy infections can lead to high levels of environmental contamination, which influences the rate of infection in susceptible hosts. Infected calves and lambs may shed 10^7 oocysts g^{-1} of feces and can excrete up to 10^{10} oocysts over 14 days.[46] *Cryptosporidium muris* has been isolated from wild mice in the UK,[48] but the role of rodents in transmission of cryptosporidiosis is still unclear.

Although foodborne transmission is a distinct possibility, there is little documentation of such transmission. As with other enteric protozoa, there is a potential for transmission by foodhandlers who are excreting oocysts. Direct incrimination of foodborne transmission of *Cryptosporidium* is hampered by the lack of culture techniques for the recovery of small numbers of oocysts. Epidemiological evidence points to the consumption of certain foods (raw sausage, offal, and milk) as a risk factor.[43,44,45,99] Tripe has been suspected as a source of infection.[182] Raw meat may be a potential source of human infection through cross-contamination at abattoirs where large numbers of oocysts have been detected in untreated effluent.[138] Acute gastroenteritis occurred in a group of people who had consumed chicken salad contaminated with *C. parvum*.[21] Consumption of raw milk has also been incriminated in outbreaks in the UK,[257,284] Poland,[141] and Russia.[218] In one outbreak in the United States (US) cryptosporidiosis was reported in children drinking school milk and linked to a defective on-farm pasteurization plant.[102] Outbreaks of cryptosporidiosis have been reported among people drinking freshly pressed, unpasteurized cider.[174,177] Fallen apples contaminated with animal manure have been incriminated as the presumed source of infection.

Cryptosporidium oocysts are prevalent in surface waters.[119] Contamination is frequently due to human or animal feces.[217] Adverse weather conditions such as heavy rain and floods can cause sewage overflow and increase agricultural and urban runoff resulting in water contamination.[156] Accidental pollution and heavy rainfall, especially following the application of manure to agricultural land, can rapidly increase the levels of contamination. In many instances, the species of *Cryptosporidium* is not identified, so the infectivity for humans is not determined. Concern has increased following a report that *C. parvum* could be experimentally transmitted to aquatic lower vertebrates (fish, amphibians, and reptiles) and then back to mammals.[9] Waterfowl also have been shown to excrete *C. parvum* oocysts, acting as mechanical vectors and disseminating the pathogen in the environment.[110]

Outbreaks of cryptosporidiosis associated with contaminated drinking water have been reported from a number of countries including large outbreaks in the US and UK. The largest outbreak of waterborne cryptosporidiosis was reported in Milwaukee, Wisconsin, with an estimated 403,000 people becoming ill.[169] In the UK, outbreaks of cryptosporidiosis have been reported from a number of counties associated with contaminated drinking water[100,139,214] or contaminated swimming pools.[98]

Clinical Signs. *Cryptosporidium* infection was first associated with severe diarrhea in humans but other clinical profiles are recognized.[261] The diarrhea is more profuse in immunocompromised people than in immunocompetent persons. In such severely immunocompromised persons as those with AIDS, infections are of long duration, often followed by death. Other clinical signs can include abdominal pain, nausea, vomiting, and fever. The onset of cryptosporidiosis often begins with an influenza-like illness. Nonspecific symptoms include loss of appetite, malaise, muscle pain, weakness, and headache. The infective dose can be small, approximately 1,000 oocysts or less, and the time from exposure until clinical illness can range from 5 to 21 days.

In animals, particularly young ruminants, symptoms include profuse watery diarrhea, abdominal pain, and dehydration, which may be followed by recovery and immunity to further clinical episodes. Disease is often associated with the presence of other organisms, notably enterotoxogenic *Escherichia coli*, *Salmonella*, *Clostridium perfringens*, rotavirus, and coronavirus,[57] all of which may contribute to the neonatal diarrhea complex, although evidence suggests that *C. parvum* is a primary pathogen in its own right.[8]

Methods of Detection. The epidemiological investigation of foodborne cryptosporidiosis and the detection of *Cryptosporidium* in food have been limited by the lack of efficient detection methods. At present, there are no convenient, specific tests for *Cryptosporidium* in food. Methods adapted for clinical diagnosis or used by the water industry offer the only means of detection. A variety of techniques have been developed for processing fecal specimens to obtain and identify oocysts.[58] The oocyst of *Cryptosporidium parvum* is approximately 4 μm to 6 μm in diameter. Current laboratory methods of diagnosis of clinical cryptosporidiosis rely on the direct examination of fecal smears or fecal concentration and subsequent microscopic examination. In food, and in treated or environmental samples, oocysts usually are present in low numbers, requiring concentration techniques in order to increase the chances of recovery.[149]

Concentration of oocysts from water is possible by filtration of large volumes of water using cartridge or membrane filtration, or smaller volumes using a calcium carbonate flocculation technique.[96] In food, similar concentration techniques may be applied prior to detection.[149]

Several stains have been used to differentiate oocysts from surrounding debris, including acid-fast stains, Giemsa, Gram stain, methenamine silver, Periodic Acid Shift (PAS), and others. These methods, which are summarized in Table 51–2, are generally considered to be adequate for detection of *Cryptosporidium* oocysts in samples from acute infections where many oocysts are present. Acid-fast stains are fast, relatively easy to use, accurate, and inexpensive. Most oocysts stain red, differentiating them from green-stained yeasts of the same size (4.5 μm to 5 μm diameter) and shape. Fluorescent stains alone or combined with specific anti-*C. parvum* antibody have also been used successfully. The detection limits using such techniques may be as high as 500,000 oocysts g^{-1} of feces.[271]

In human diarrheal cases, a further problem faced is the microscopic misidentification of *Cryptosporidium* as *Cyclospora*.[283] These organisms stain similarly using acid-fast stains. *Cyclospora* cysts (8 μm to 10 μm, two sporocysts with 2 sporozoites [see Section 51.2.2]) are slightly larger than *C. parvum* oocysts (4 μm to 6 μm, four sporozoites without sporocysts), but very similar in size to *C. muris* (7.4 μm × 5-6 μm). The internal structures are not clearly visible without the use of electron microscopy.

Speciation of *Cryptosporidium* is difficult, if not impossible, using these conventional techniques. A range of molecular and immunological techniques has been developed. Monoclonal antibodies (mAbs) have been produced that are specific to polypeptides, with the ability to distinguish sporozoites of *C. parvum*, which is potentially the most pathogenic species to mammals, from those of other cryptosporidia. mAbs have become the basis for alternative detection methods that involve immunofluorescence (IF) or enzyme-linked immunosorbent assays (ELISA).[222] Commercially available ELISA and IF kits, based on genus-specific monoclonal antibodies to oocyst surface proteins, are available for use in diagnostic laboratories, but at present there are no mAbs that can be used to discriminate between species of intact oocysts.[253] The detection limits of ELISAs and IF tests are similar to those stated above for the examination of stained fecal smears and depend primarily on the concentration methods employed to prepare the sample.[271,273]

DNA-based techniques, using PCR, have been investigated for use in the detection of *Cryptosporidium*.[149,151,275] Before such techniques are adopted they require rigorous testing and comparison with existing methodologies. They do, however, offer the advantage of increased sensitivity, when used in conjunction with immuno-magnetic separation (IMS).[274]

Prevention and Control. To prevent or control cryptosporidiosis, oocysts must be eliminated from the environment or rendered nonviable, or susceptible people must avoid contact with known sources of oocysts such as infected animals or people and contaminated environments.[85] Because immunocompromised patients are at greater risk from cryptosporidiosis, specific measures have been recommended.[140] These include minimizing oral exposure to water from lakes, streams, and public swimming pools, and treating drinking water by either boiling or by using a filter capable of removing particles greater than 1 μm in size. The safety of bottled water varies from supplier to supplier. Heating water to temperatures above 72.4° C inactivates *C. parvum* oocysts.[84]

A number of control measures are used for the treatment of potable water to render it safe for drinking; however, unless prepared specially, no water is totally clear of particles under 10 μm and the addition of chlorine as a final disinfectant for bacteria has no effect on oocysts. Indeed, few disinfectants have been found effective within a relatively short period of exposure and the application of these chemicals is relatively impractical. A review of water purification methods for removal of oocysts is given by Badenoch.[14] The use of slow sand filtration and chemical flocculation in the water treatment process can increase the removal of oocysts.

Table 51–2 Summary of Laboratory Methods Available for Detection of *Cryptosporidium*

Technique	*Method(s)*	*Sensitivity (oocysts g^{-1})*	*Speciation*	*Use*
Smear/Stains	Ziehl Neelson (ZN)	>100,000	Based on morphology	Clinical diagnosis
	Auramine Phenol (AP)	>50,000		
Concentration	Flotation-NaCl-Sucrose Formalin-ethyl acetate	>500,000	Based on morphology	Clinical diagnosis
	Flotation + stain (eg, Sucrose + AP)	>5,000	Based on morphology	Clinical diagnosis
	Cartridge/membrane filtration	N/A	Based on morphology	Monitoring of water and environmental samples
Immunological	IFA	>50,000	Based on morphology	Clinical diagnosis
	ELISA	>50,000	Not possible	Clinical diagnosis
	Sample concentration and immunofluorescence	>5,000	Based on morphology	Clinical diagnosis
	Flow cytometry	N/A	Based on morphology	Monitoring of water and environmental samples; tracing asymptomatic carriers
Biophysical and Biochemical Techniques	Isoenzyme analysis	N/A	Based on banding patterns	Isolate characterization
	Two-dimensional electrophoresis	N/A	Based on banding patterns	Isolate characterization
DNA-Based Techniques	PCR	<100	Specific probes	Monitoring of water and environmental samples; tracing asymptomatic carriers
	RFLP analysis	N/A	Based on banding patterns	Isolate characterization
	RAPD-PCR	N/A	Based on banding patterns	Isolate characterization
	DNA sequencing	N/A	Based on analysis of sequence data	Isolate characterization

IFA–Immunofluorescence assay; ELISA–Enzyme-linked immunosorbent assay; PCR–polymerase chain reaction; RFLP-Restriction fragment length polymorphism; RAPD-PCR—Random Amplified Polymorphic DNA–Polymerase Chain Reaction
N/A—not applicable

Source: Copyright © 1998, Ministry of Agriculture, Fisheries and Food, UK.

Freezing or adequate cooking of foods will destroy oocysts and thus limit foodborne transmission. Personal hygiene and sanitation can reduce oocyst numbers. Thorough washing of hands and the removal of soil from fresh vegetables can reduce the risk of hand-to-mouth or foodborne ingestion of oocysts.

Conclusion. Outbreaks of cryptosporidiosis due to foodborne transmission have rarely been reported but the high prevalence of infection in man and animals, together with the high infectivity of *Cryptosporidium parvum* and fecal-oral route of transmission, offer the potential for foodborne infections. The immunocompromised are at greatest risk and should take steps to limit exposure. The greatest potential risk comes from ingestion of fresh, raw, or uncooked foods. Avoidance of contamination of vegetables and fruits in the field, strict hygiene measures involving handling of food, and the susceptibility of the organism to freezing and cooking offer effective means of control. In contrast, waterborne transmission appears relatively common and can result in large-scale outbreaks of disease. Improvement in water catchment may reduce the potential health risks associated with the presence of oocysts in water supplies, but probably would not eliminate the problem. No single conventional treatment process is considered able to remove oocysts entirely but effective flocculation and filtration may reduce their numbers to a low level of hazard.[117] The zoonotic potential of cryptosporidiosis necessitates strict personal hygiene especially when handling young farm livestock and especially where children are concerned.

51.2.2 *Cyclospora*

History. *Cyclospora* are coccidian parasites reported from a number of animals that have assumed importance more recently in man, with worldwide reports of the presence of "cyanobacterium-like bodies" in the feces of immunocompetent and immunocompromised patients with diarrhea.[190] These organisms are now recognized as *Cyclospora cayetanensis* and are a cause of protracted diarrhea in humans. Parasites of this genus were first reported in the late 19th century and since have been described in insectivorous mammals,[80,92,93] nonhuman primates,[242] and snakes.[195,200,201]

It is thought that initial cases of infection in man were either reported as unidentified coccidian parasites[11] probably misidentified as *Isospora*,[215] *Cryptosporidium*,[244] or described as cyanobacterium-like organisms.[157]

Life Cycle. The life cycle in animals typically is coccidian and is likely to be similar in humans. Infection commences with ingestion of the sporulated oocyst (containing 2 sporocysts each with 2 sporozoites). Development occurs within the gut epithelium with asexual development (merogony) producing merozoites. The number of merogony stages varies, but ultimately the last meront stage produces merozoites that develop into gametocytes to product a zygote and then oocyst. Oocysts are passed in the feces and undergo further development (sporogony) outside the body to produce the final infective, fully sporulated oocyst stage. Sporulation is prolonged, taking from 2 weeks to 4 to 6 months depending on temperature.[244]

Prevalence in Man and Animals. Although *Cyclospora* appears to be widely distributed throughout the world, the prevalence of infection in man and animals worldwide is unknown. It has been reported in North, Central, and South America, Eastern Europe, India, South Africa, and Southeast Asia.[243] In Canada and the US, approximately 1 in 1,000 human fecal samples from patients being investigated for gastrointestinal abnormalities were found to be positive[25,187] although the prevalence in the general population was considered to be generally lower. In the UK, *Cyclospora* was identified in 0.1% of 1,333 human fecal samples from healthy adults.[51] Prevalence rates of 11% have been reported among nonnative adults and children in Nepal during the rainy season.[125] In human immunodeficiency virus (HIV)-infected Haitian adults with chronic diarrhea, 11% had *Cyclospora* infection.[194]

Epidemiology and Transmission. An outbreak of *Cyclospora* occurred in a hospital in the US that pointed to water as a likely source of infection.[244] Other outbreaks of infection via drinking water have been reported,[208] and consumption of untreated water during foreign travel has been incriminated as a factor in the epidemiology of the disease.[125] More recently there have been several reports of foodborne transmission associated with the consumption of fruit in North America. A large outbreak of cyclosporiosis in the US was associated with the consumption of imported raspberries.[122] Mesclun (a mixture of various types of baby leaves of lettuce) was suspected of being the vehicle for one or possibly two outbreaks in the US in 1997.[178] The consumption of basil was suspected as a source of infection at a luncheon in Virginia.[179] Most cases of cyclosporiosis in the UK appear to be linked with foreign travel.[19]

Clinical Signs. The most typical signs of cyclosporiosis infection in man are watery diarrhea with nausea, vomiting, anorexia, bloating, abdominal cramps, fatigue, and malaise.[11,125,235,243] The incubation period ranges from 2 to 11 days.[243] Among immunocompromized hosts, *Cyclospora* infection has occurred predominantly in HIV-infected individuals. Clinical illness in these patients is prolonged and severe[125] and is associated with a high rate of recurrence.[194,236]

Methods of Detection. In fresh fecal samples, *C. cayetanensis* appears as nonrefractile cysts, 8 to 10 µm in diameter. The cyst contains a pale green morula composed of refractile, membrane-bound globules in a rosette configuration.[157] Sporulated oocysts contain two sporocysts each with two sporozoites. The internal structures are not clearly visible without the use of electron microscopy. *Cyclospora* oocysts may be confused with *Cryptosporidium* oocysts unless measurements are taken with a micrometer. The organisms stain similarly using acid fast stains. *Cyclospora* cysts are slightly larger than

C. parvum oocysts (4 μm to 6 μm); those from animals may vary in size up to 17 μm to 18 μm.[92]

Methods used for screening water samples for *Cryptosporidium* should prove equally effective for *Cyclospora* once suitable fluorescent-labeled monoclonal antibodies have been developed. Detection of *Cyclospora* on food, particularly fruit, is difficult even though methods for detecting protozoan contamination exist. Fruit juice interferes with DNA methods and fruit debris interferes with microscopy.[130]

Prevention and Control. Precautions similar to those recommended for cryptosporidiosis (see Section 51.2.1) should be taken wherever possible. In third-world countries with poor sanitation, one should only consume water that is either boiled or bottled. As most reports of cyclosporiosis occur following consumption of fresh fruit and salads, these should be washed thoroughly and food handlers should ensure good personal hygiene.

Conclusion. Based on recent reports of both waterborne and foodborne outbreaks, it is evident that *Cyclospora* is a rapidly emerging pathogen and cause of diarrheal illness in man. At this stage, little is known of potential sources and mode of contamination with the organism. The greatest potential risk appears to be from contaminated drinking water, particularly on foreign travels, and from fresh produce. As with other infections, produce to be eaten raw should be thoroughly washed, including salad vegetables and certain fruits if they have been grown in conditions exposed to fecal contamination.

51.2.3 *Sarcocystis*

History. *Sarcocystis* is one of the most prevalent parasites of livestock and infects mammals, including man, birds, and lower vertebrates.[78] About 130 different species have been reported. Controversy still exists over the generic and specific taxonomy such that the nomenclature remains confusing.[254] The parasites derive their name from the intramuscular cyst stage (sarcocyst) of the life cycle, which for many years after the discovery of these parasites was the only stage known. Cysts are found in virtually all skeletal muscles of the body including the tongue, esophagus, and diaphragm, as well as in cardiac muscle and to a lesser extent in the brain. *Sarcocystis* spp. are obligatory heteroxenous parasites, ie, both an intermediate and a definitive host need to be present for the continuation of the life cycle. Asexual reproduction (merogony) and cyst formation occur in the intermediate host, while sexual reproduction (gametogony) and oocyst maturation (sporogony) occur in the definitive host (Figure 51–2). Intermediate hosts of *Sarcocystis* are usually herbivores or omnivores that become infected by ingesting sporocysts with contaminated food or water. Definitive hosts are carnivores, which become infected by ingesting cysts contained in muscle or nervous tissue of intermediate hosts.

Most *Sarcocystis* infecting man and domestic animals are species-specific for their intermediate hosts and family-specific for their definitive hosts.[254] Intermediate as well as definitive hosts can be infected by several different *Sarcocystis.* Livestock can be intermediate hosts for several *Sarcocystis* spp. of different pathogenicity (Table 51–3). In man, two species of *Sarcocystis* have been described with varying pathogenicity. Foodborne transmission therefore occurs through the ingestion of raw or undercooked meat containing mature sarcocysts.

Life Cycle. Sarcocysts present in the muscle of the intermediate host contain large numbers of spindle-shaped bradyzoites or cystozoites (the terms being synonymous with merozoites) that appear dormant until the cyst is ingested. Following digestion by the final host, bradyzoites liberated from the cyst become active and enter cells in the intestinal lamina propria in which they develop into the sexual stages of microgametes or macrogamonts. Macrogamonts are fertilized by the sperm-like microgametes producing zygotes, which then develop into oocysts. Oocysts sporulate in the intestine, forming two sporocysts that each contain four sporozoites. Often, the thin oocyst wall ruptures during passage in the feces, with individual sporocysts shed by the infected person or animal. These sporocysts are infectious only for the same species of intermediate host that served as the original source of infection. After ingestion of sporocysts, sporozoites are liberated in the gut and find their way to arterioles throughout the body, from which they enter cells and undergo asexual multiplication, producing merozoites. These in turn leave the host cell and travel in the bloodstream to the capillaries, in which they enter new host cells and again undergo asexual multiplication to produce a second generation of merozoites. When these become mature and leave the host cell, they enter skeletal and cardiac muscle cells from which they give rise to the cyst stage (containing bradyzoites).

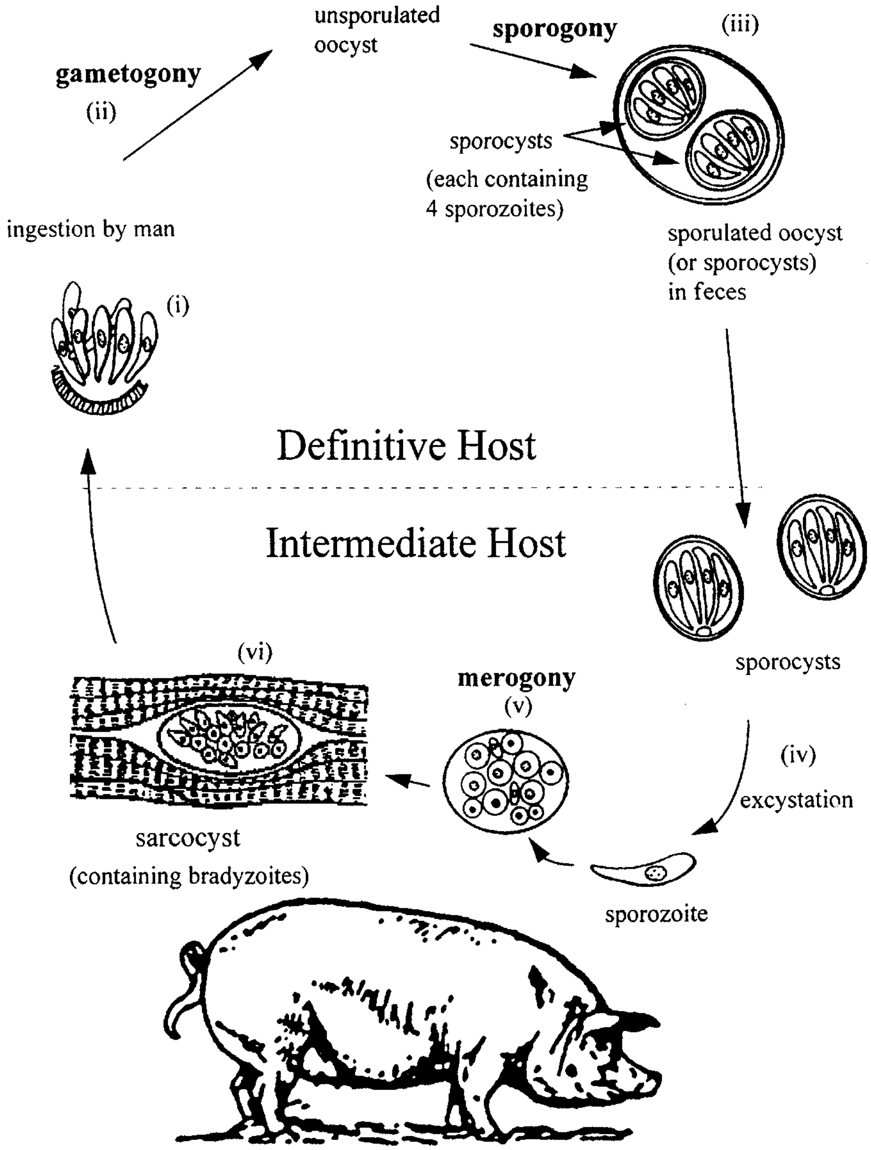


Figure 51–2 Life Cycle of *Sarcocystis suihominis*. Ingestion of sarcocysts (i), present in the muscle of the intermediate host, results in the release of bradyzoites in the small intestine of the definitive or carnivorous host. The bradyzoites penetrate the mucosa of the small intestine and develop into macro and microgametocytes [gametogony (ii)], which fuse to form zygotes released into the gut lumen as unsporulated oocysts. Oocysts sporulate in the intestine forming two sporocysts each with 4 sporozoites (iii). Rupture of the thin-walled oocysts in the intestine leads to passage of either oocysts or sporocysts (iv) into the feces. Sporozoites are released into the intestine following ingestion by the intermediate host from where they travel via the bloodstream to the blood vessels where they undergo two cycles of asexual development in the lining of endothelial cells [merogony (v)] before entering cardiac or skeletal muscle and forming sporocysts (vi) containing large numbers of bradyzoites.

Prevalence in Man and Animals. There are two known species of *Sarcocystis* that infect man: *S. hominis* (*S. bovihominis*) and *S. suihominis* (Table 51–3). There is evidence to suggest that oocysts of these species were previously referred to as *Isospora hominis*.[1] Humans also serve as the accidental intermediate host for several unidentified species of *Sarcocystis*.[17,78] Intestinal sarcocystosis in man, associated with clinical signs of gastrointestinal upset, appears to be more common in Europe than in other continents, with reports in Czechoslovakia,[105] France,[60] Germany,[91,132] and Poland.[202] Levels of in-

Table 51–3 *Sarcocystis* of Man and Domestic Animals

Intermediate Host	*Definitive Host*	*Sarcocystis*	*Pathogenicity (intermediate host)*
Cattle	Man, primates	*S. hominis (S. bovihominis)*	low
	Dog, wolf, fox	*S. cruzi (S. bovicanis)*	high
	Cat	*S. hirsuta (S. bovifelis)*	low
Sheep	Dog, fox	*S. tenella (S. ovicanis)*	high
	Dog, fox	*S. arieticanis (S. ovicanis)*	intermediate
	Cat	*S. gigantea (S. ovifelis)*	nonpathogenic
	Cat	*S. medusiformis (S. ovifelis)*	nonpathogenic
Pig	Man, primates	*S. suihominis (S. hominis)*	high
	Dog, cat	*S. miescheriana (S. suicanis)*	high
		S. porcifelis (S. suifelis)	unknown
Goat	Dog, fox	*S. capracanis*	high
	Dog	*S. hircicanis*	intermediate
	Cat	*S. moulei*	nonpathogenic
Horse	Dog	*S. bertarmi*	low
Chicken	Unknown	*S. horvathi*	unknown

fection in man may reflect cooking habits and preferences for undercooked meat.

There are three species of *Sarcocystis* affecting cattle. Only one species, *S. hominis* (*S. bovihominis*), is infective for man. Examination of various tissues suggests that most cattle worldwide are infected with *Sarcocystis*,[78] with *S. cruzi* (*S. bovicanis*), which is infective to dogs, the most prevalent. Infection of cattle with *S. hominis* appears to be fairly low in many countries, with the possible exception of Germany where up to 63% of cattle have been shown to be infected.[228] *S. hominis* can only be differentiated from *S. hirsuta* (*S. bovifelis*) by electron microscopy, and thus information on the prevalence of the former species may be misleading or inaccurate. Almost all adult sheep can be infected with *Sarcocystis* but none of these species are infective for man.[78] Of the four species, two infect dogs, *S. tenella* and *S. arieticanis* (*S. ovicanis*), and two infect cats, *S. gigantea* and *S. medusiformis* (*S. ovifelis*). The overall prevalence of *Sarcocystis* in pigs is low, between 3% and 36% worldwide.[78] Three species occur in pigs, one of which, *Sarcocystis suihominis*, is infective for man. This species has been reported in European countries with the highest prevalence in Germany.[22] The other two species found in pigs infect either dogs, *S. miescheriana* (*S. suicanis*), or cats, *S. porcifelis* (*S. suifelis*).

Epidemiology and Transmission. In most cases, humans acquire gastrointestinal sarcocystosis by ingesting raw or undercooked meat from cattle or pigs harboring mature *S. hominis* or *S. suihominis* cysts. Macroscopic cysts of *Sarcocystis* are often removed at meat inspection, although these cysts are usually of no significance to man. Some meat animals such as camels, llamas, water buffalo, and yaks harbor *Sarcocystis* in which the final host is unknown. Man may also harbor sarcocysts in striated muscle and therefore serve as the intermediate host.[17] The species and life cycle are unknown but are often collectively referred to as *S. lindemanii*. In a survey of 100 human tongue tissues taken at autopsy in Malaysia and examined histopathologically, a 21% incidence of *Sarcocystis* was found.[280] Based on the knowledge of all other *Sarcocystis* life cycles, infected human tissue would have to be eaten by a carnivore to complete the life cycle. Because there is no known predatory or scavenging cycle in nature in which human tissues are eaten regularly by carnivores, humans most likely become infected accidentally by ingestion of water contaminated by the feces of a carnivore.

Clinical Signs. Human volunteers became infected and shed oocysts in their feces following the ingestion of raw beef containing *S. hominis*,[10,220] although only one volunteer became ill. The clinical signs that appeared 3 to 6 hours after eating the beef included nausea, stomachache, and diarrhea. In contrast, volunteers who ate raw pork infected with *S. suihominis* also became infected and shed oocysts but had much more dramatic symptoms that appeared 6 to 48 hours after ingestion.[123,220] These clinical signs included bloat, nausea, loss of appetite, stomachache, vomiting, diarrhea, difficulty breathing, and rapid pulse. Volunteers who ate well-cooked meat from the same pigs developed no clinical signs.[123] *Sarcocystis* may be responsible for several idiopathic diseases in man including cardiac diseases such as cardiomyopathy and myocarditis[13,116] and rheumatic diseases.[116] It also has been suggested that *Sarcocystis* may be associated with muscle aches and fatigue as part of the chronic fatigue syndrome (CFS).[199]

In animals, most pathogenic *Sarcocystis* cause disease only in their intermediate hosts and not in their definitive hosts. Carnivores fed meat infected with a number of species of *Sarcocystis* shed sporocysts in their feces but usually appear clinically normal. Little is known about the mechanisms that cause pathogenicity and not all species are pathogenic in their intermediate hosts. Species that infect ruminants appear to be the most pathogenic with those transmitted by dogs or primates generally more pathogenic than those transmitted by cats (Table 51–3). The severity of clinical disease depends on the dose of ingested sporocysts and the immune status of the host. The uptake of a high number of sporocysts of a pathogenic *Sarcocystis*, in particular of *S. cruzi* in cattle or *S. tenella* in sheep, can lead to acute sarcocystiosis in previously uninfected animals that have not developed immunity. Of the three species infecting cattle, *S. cruzi* is the most pathogenic, causing a variety of symptoms including fever, anorexia, anemia, weight loss, weakness, muscle twitching, prostration, depressed milk yield, hypersalivation, neurological signs, and death depending on the number of sporocysts ingested.[78] In pregnant animals, acute sarcocystiosis frequently results in fetal death, abortion, or premature birth of the offspring.[87] Animals surviving a primary *Sarcocystis* infection usually acquire immunity that protects them against acute disease after challenge with the homologous species, but not after infection with a pathogenic heterologous species. Chronic sarcocystiosis of domestic livestock can result from the ingestion of a low dose of sporocysts and can cause economic losses due to reduced quality and quantity of meat. Additional economic losses occur with those species that form macroscopic cysts resulting in condemnation of whole carcasses or affected parts after slaughter.

Methods of Detection. Determination of *Sarcocystis* in meat can be made by direct observation of macroscopic cysts or microscopic examination of histological sections. Meat also can be ground and artificially digested with a mixture of pepsin and hydrochloric acid at 37° C for 1 hour, centrifuged, and the pellet examined microscopically for the presence of bradyzoites.[78]

Presumptive diagnosis of human intestinal sarcocystiosis is based on clinical signs and a medical history indicating ingestion of raw or undercooked meat. Definitive diagnosis would require identification of sporocysts in the feces. Sporocysts can be identified by concentration from fecal material, using a sucrose flotation medium and examination using bright field microscopy. Sporocysts of *S. hominis* are shed 14 to 18 days after ingesting beef and average 14.7 μm by 9.3 μm in size. Those of *S. suihominis* are shed 11 to 13 days after ingestion of pork and average 13.5 μm by 10.5 μm.

Clinical symptoms of acute sarcocystiosis in ruminants are nonspecific, and diagnosis, therefore, is currently based on the histological detection of meronts at postmortem examination. In abortions, meronts may be found in maternal tissues of the placenta, but are not found consistently in fetal membranes or tissues. In chronic sarcocystiosis, cysts can be found microscopically in skeletal or heart muscle. While cystozoites of different *Sarcocystis* are too similar morphologically to be differentiated from each other, some *Sarcocystis* can be identified in stained histological sections or squash preparations based on the morphology of their cyst wall. In many cases, a species-specific diagnosis of *Sarcocystis* cysts is not possible and requires the use of electron microscopy.

To improve the sensitivity and specificity of histological detection of *Sarcocystis* stages, several immunohistochemical methods have been developed.[118,259] However, polyclonal antisera derived from animals that have been immunized with any species of *Sarcocystis* show a high cross-reactivity with antigens of heterologous *Sarcocystis*. It is, therefore, currently not possible to differentiate between infections with pathogenic and nonpathogenic *Sarcocystis* in intermediate hosts.

Prevention and Control. Human infection with intestinal sarcocystiosis can be prevented by ensuring that meat is cooked thoroughly or frozen to kill bradyzoites in cysts. To interrupt the life cycle and prevent infection of livestock, carcasses, meat scraps, offal, or other raw or undercooked tissues should not be available to domestic or wild carnivores in areas where livestock are raised.

Conclusion. Although a widespread and common infection in domestic animals, transmission to man through ingestion of infected meat is a relatively rare occurrence. Species of *Sarcocystis* are highly host-specific, and only two species are known to infect man. The greatest potential risk therefore comes from ingestion of raw or uncooked beef or pork. Providing meat is well cooked, *Sarcocystis* should not present a serious foodborne risk. Residents or visitors to countries where raw or undercooked meat is served should beware or suffer the consequences.

51.2.4 *Toxoplasma*

History. *Toxoplasma gondii* is an obligate intracellular parasite with a typical coccidian life cycle in which gametogony and oocyst production occur in domestic and wild cats.[83] The parasite differs from other coccidia, however, in that it shows a complete lack of specificity for hosts and tissues during its asexual phase and can infect a wide range of animals including man. The parasite has worldwide distribution and is of both medical and veterinary importance.

Life Cycle. Although the parasite was first identified in 1908,[183] it was not until 1970 that its full life cycle was elucidated.[94,127] Following ingestion of tissue cysts by the definitive feline host, bradyzoites are liberated and enter the epithelium of the small and large intestines in which they undergo asexual reproduction (merogony) passing through five distinct morphological phases before sexual reproduction (gametogeny) begins, mostly in the epithelial cells of the small intestine.[74,153] After fertilization, zygotes develop into oocysts that enter the lumen of the intestine and are shed unsporulated in the feces (Figure 51–3).

The time from ingestion of cysts until oocysts are shed is 3 to 10 days and shedding usually lasts 7 to 21 days.[85] The oocysts are not immediately infectious, requiring 2 to 3 days of further development (sporogony). The sporulated oocyst contains two sporocysts each with four sporozoites. How often a cat is capable of shedding oocysts is not known. Cats can repeat oocyst shedding after reinfection, although the number shed is far less than during the primary infection.[68] Older cats usually do not shed oocysts unless immunocompromised or infected with some other intercurrent disease. When either oocysts or tissue cysts are ingested, sporozoites or bradyzoites enter the tissues of a nonfeline host and transform into the tachyzoite stage. Tachyzoites undergo repeated, rapid multiplication by a process called *endodyogeny* (two progeny form within the parent tachyzoite) until, influenced by the host's immune system, the multiplication rate slows and the tachyzoites transform into bradyzoites. These become surrounded by a cyst wall and remain dormant but infectious in the tissue for long periods of time, sometimes for the life of the host. Bradyzoites transform back into tachyzoites when the cysts are ingested by another host or when the immune system of the current host is suppressed by malnutrition, disease, or medications.

Prevalence in Man and Animals. *Toxoplasma* is widespread among man and animals and is possibly the most widespread and prevalent protozoan parasite on earth,[83] although the reported prevalence varies greatly. It has been estimated that more than 500 million people are infected with it.[75] In the US and the UK, estimated infection rates in man are between 20% to 40%, but are much higher in European countries with rates ranging from 50% to 80%.[72] Infection appears to be more common in warm climates and low-lying areas, probably relating to climatic conditions favoring oocyst sporulation and survival.[95,266,288] In Europe, congenital toxoplasmosis is found in 1% to 6% of newborns and is probably the most serious form of *Toxoplasma* infection. In the US, more than 3,000 babies are born with toxoplasmosis each year; of these, 5% to 15% die, 8% to 10% have brain and ocular lesions, 10% to 13% have visual damage, and the 58% to 72% who are normal at birth later develop active infections.[75] Rates of congenital toxoplasmosis worldwide are estimated at 0.1% to 0.8% of live births.[216] Toxoplasmic encephalitis is now a major opportunistic infection in immunocompromised individuals and it is estimated that 30% of AIDS patients seropositive to *T. gondii* will develop toxoplasmic encephalitis.[59]

In a number of serologic surveys conducted in the US, 25% of 4,871 cats examined were seropositive for *Toxoplasma.*[75] Seropositivity increases with age and varies according to lifestyle, with rates being higher in feral cats than in domestic cats.[67] The prevalence of active shedding of oocysts by cats at any given time is thought to be as low as 1%.[94] This figure can be misleading when considering the potential of cats to spread infection because of the large number of oocysts shed and the ability of oocysts to remain viable for at least 410 days.[288]

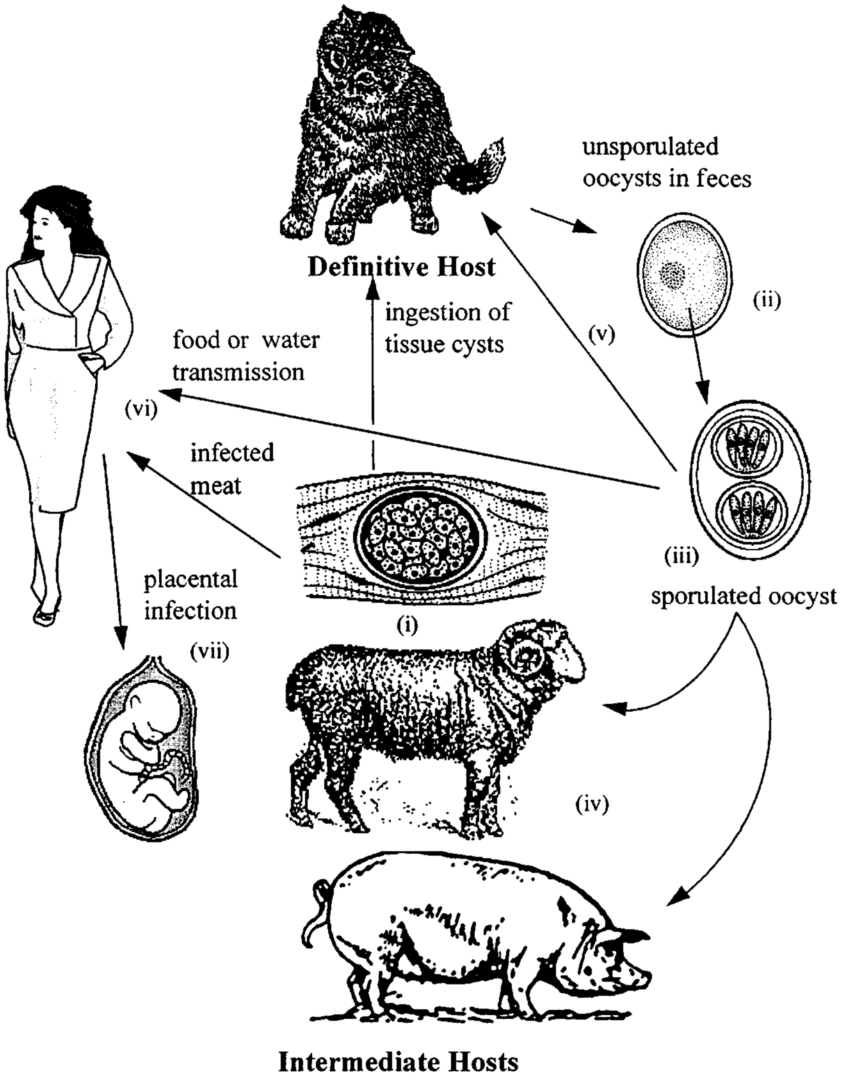


Figure 51–3 Life Cycle of *Toxoplasma gondii.* Following ingestion of tissue cysts (i) by the definitive feline host, liberated bradyzoites transform into tachyzoites and undergo asexual reproduction (merogony) followed by sexual reproduction (gametogony) within epithelial cells of the intestine. After fertilization, unsporulated oocysts (ii) enter the lumen of the intestine and are shed in the feces. After a period of 2 to 3 days, the oocysts sporulate (sporogony) and contain 2 sporocysts each with 4 sporozoites (iii). Ingestion of oocysts by nonfeline hosts (iv) leads to the release of sporozoites, which transform to tachyzoites that undergo rapid multiplication (endodyogeny) producing large numbers of bradyzoites that become surrounded by a cyst wall and remain dormant but infectious. Cats may be infected by ingestion of the sporulated oocysts (v) passed in the feces of other cats. Man becomes infected either by ingestion of the sporulated oocyst through contamination by cat feces or by ingestion of undercooked meat containing tissue cysts (vi). Infection during pregnancy (vii) can lead to congenital infection of the fetus or abortion.

An extensive review has been undertaken on the worldwide prevalence of *Toxoplasma* in food animals based on both serological testing as well as parasite isolation from tissues.[75] In sheep, the prevalence of antibodies increases with age with the level in ewes more than twice that of young lambs. Prevalence appears higher on farms where abortions occur than in normal flocks. Viable *T. gondii* cysts have been recovered from as many as 67% of sheep, mainly from the diaphragm, muscle, and brain. Of all the meat animals, a relatively high percentage of pigs have *Toxoplasma* antibody. Up to 30% of pigs were seropositive and cysts have been isolated from the diaphragm and common cuts of meat. *T. gondii* is rarely isolated from bovine tissues. Serological prevalence varies depending on the serological method used. The actual prevalence may be lower than the reported seroprevalence because of nonspecific cross-reactions with other protozoa, particularly the closely related protozoan parasite, *Neospora caninum* in cattle, dogs, and other animals.[53,76,234]

It has been argued that the estimated levels of infection of lamb, pork, and beef should not be based on serological findings for several reasons. These include strain variation in infectivity, differences in the kinetics of the host's serological response to infection, cross-reactivity, and genetic or other unknown factors imparting resistance to persistent infection in the host.[85]

Epidemiology and Transmission. Humans can acquire toxoplasmosis by ingestion of sporulated oocysts or viable tissue cysts in meat from infected animals. Although a major source of infection of animals is thought to result from contamination of the environment with oocysts shed in cat feces, the extent of human infection resulting from this route is not known. Feces are deposited widely in gardens, fields, playgrounds, buildings, feed troughs, and almost any other location available to cats. The oocysts are easily dispersed by wind and water; by transport hosts such as earthworms, slugs, and insects; by birds or the feet of humans and animals; or indirectly via fomites. They can remain infectious for a year or more if protected from temperature extremes, intense sunlight, and drying. Infection by ingestion of oocysts was the only explanation for the relatively high prevalence of *Toxoplasma* antibody in vegetarians in India.[210]

Although transmission of *Toxoplasma* via contaminated water is a distinct possibility, few reports exist.[20] In contrast, foodborne transmission by ingestion of tissue cysts in raw or undercooked meat from a variety of livestock and game animals has been well documented as a major source of human infection.[75,83] Encysted *T. gondii* are killed at temperatures above 60° C.[77] Among meat-producing animals, pigs, sheep, and goats commonly harbor tissue cysts, whereas cattle, as discussed earlier, appear to be relatively resistant and clear of infection and beef is not generally regarded as an important source of human infection.[83] Epidemiological studies suggest that in many countries ingestion of rare meat may be more important than contact with cat feces.[72] A group of students who ate undercooked hamburgers containing meat of undetermined origin developed acute toxoplasmosis 8 to 13 days later, as did another group of 110 people who ate undercooked meat.[85] Cultural habits are an important factor in the epidemiology. Several outbreaks have been associated with specific cultural preference for undercooked meat rather than mishandling or accidental undercooking. In Russia, significantly more *Toxoplasma* seropositive people were identified among women who tasted raw minced meat during cooking than among those who did not taste raw meat.[170] In France, 84% of French women have antibodies to *T. gondii* compared with 30% of women in the UK and US. The higher incidence in French women may be due to the habit of eating raw or undercooked meat. In Hawaii, a higher prevalence of infection was found among Hawaiians and Filipinos who ate raw meat (including pork) than among Caucasians and Japanese who thoroughly cooked meat.[264] In France, 204 children became infected while they were hospitalized and fed meals of very rare or raw meat.[62] Milk from infected goats has also been implicated as a source of infection.[211,225]

Occupational risks of infection may include certain farming practices, and pregnant women and other people in special risk groups should avoid contact with sheep at lambing time.[36]

Cats become infected by ingesting tissues from animals harboring cysts of *Toxoplasma* containing cystozoites or bradyzoites, by ingesting tachyzoites present in the tissues of acutely infected prey animals such as rodents, or by ingesting oocysts shed in the feces of other cats.

As with human vegetarians, herbivores must acquire *Toxoplasma* infection from oocysts. The prevalence of *Toxoplasma* antibody in herbivores such as cattle and sheep is a good indicator of the distribution of cat feces in the environment.

Clinical Signs. Clinical signs of toxoplasmosis can be highly variable because *Toxoplasma* can infect virtually every organ in the body. In humans, toxoplasmosis usually is asymptomatic or associated with mild symptoms, often only characterized by lymphadenopathy involving the posterior cervical lymph nodes. Acute infections can cause anemia, dermomyositis, encephalitis, enteritis, fever, hepatitis, lymphadenitis, myocarditis, placentitis, pneumonitis, retinochoroiditis, skeletal myositis, tynosynovitis, tonsillitis, and vasculitis.[83] The interval from exposure to clinical illness is quite variable. Generally, clinical signs are manifested from a week to a month after exposure. Duration, severity, and outcome generally vary according to the immunological state of the host. In immunocompromised individuals (eg, AIDS patients), *T. gondii* infections can be severe with cerebral involvement.[137] Perhaps the greatest significance is as a cause of congenital infection and abortion in pregnant women.[70] The fetus is thought to become infected via the placenta as a consequence of parasitemia in the mother. Usually the earlier in pregnancy infection is acquired, the greater the extent of lesions in the fetus will be. In animals, clinical signs are not usually seen in the cat but can be severe in intermediate hosts. In sheep, abortion, stillbirth, or birth of weak lambs may occur depending on when infection of the pregnant ewes occurs. Infection in mid pregnancy (60 to 90 days) usually results in abortion.[36] Ewes infected during the later stages of pregnancy (after 110 days), can lead to stillborn or weak lambs at full term.

Methods of Detection. Serological tests used in the diagnosis of toxoplasmosis include the Sabin and Feldman Dye test (DT),[224] complement fixation test (CFT), indirect fluorescent antibody test (IFAT),[42] ELISA,[260] and commercially available indirect hemagglutination test (IHA), and latex agglutination test (LAT).

Clinical signs and pathological changes in placenta and aborted fetuses of ruminants are not pathognomonic for toxoplasmosis, and detection of *Toxoplasma* tachyzoites by histological methods is not always easy, especially in autolyzed material. Impression smears stained with Giemsa or Romanowsky[69] or PAS[71] may allow identification of tachyzoites or tissue cysts. Diagnosis may be confirmed by isolation of viable parasites by mouse inoculation with tachyzoites or cysts or by demonstration of parasite stages by immunohistochemical methods.

Recently diagnostic methods based on detection of *T. gondii*-specific DNA by PCR have been developed. These have been based upon the p30 gene and can detect a single organism in the presence of a million host cells.[226] This PCR has also been applied to tissues from sheep (brain, amnion/chorion, peritoneal fluid, and cotyledons).[277] Other PCRs have been developed based on sequences of ribosomal DNA[113] and the B1 gene.[140] A study comparing the p30 and the B1 PCRs found that the B1 PCR was more sensitive, it gave fewer false negatives, and the results correlated closely with conventional mouse inoculation methods.[270]

Prevention and Control. Any measures that reduce or eliminate contamination of the environment with oocysts from cat feces will help prevent toxoplasmosis. Domestic cats should be prevented from hunting birds and rodents. They should be fed thoroughly cooked food (dry or canned). Litter trays should be used in the home and emptied daily. Gardeners handling soil should wear gloves and wash their hands thoroughly before eating or handling food. Cats on farms should be prevented from entering feed-storage facilities and animal quarters.

All meat should be cooked thoroughly before eating. Cooking at 60° C or higher for 3½ minutes or longer renders *Toxoplasma* cysts noninfectious.[77] It is important to know the actual temperature in the core of the meat, not just the surface temperature. Microwave ovens should not be used for initial cooking because of the uneven distribution of heat resulting in hot and cold spots.[85] Freezing to –20° C also kills some strains of *Toxoplasma* but is less dependable than cooking. Salting, curing, and pickling also kill cysts present in meat.[281]

Irradiation of meat at 0.5 kGy or greater renders *Toxoplasma* cysts noninfectious.[73]

Finally, water from lakes, ponds, streams, or other untreated sources should be boiled before drinking or being used to wash food.

Conclusion. Infection with *Toxoplasma gondii* is one of the most common of parasitic infections of man and other warm-blooded animals. Foodborne transmission through ingestion of raw or undercooked meat or other animal produce such as milk is well documented. Adequate cooking kills the organism as does pasteurization. Contact with cat feces should be avoided, especially in the immunocompromised and in women during pregnancy. Contaminated water presents a risk and steps should be taken wherever possible to avoid consumption of untreated drinking water.

51.3 FLAGELLATE PROTOZOA (MASTIGOPHORA)

51.3.1 *Giardia*

History. *Giardia* infections are frequently overlooked in domestic animals despite the isolation of the protozoan from a variety of mammalian, avian, reptilian, amphibian, and fish hosts. The host specificity of *Giardia* is still not known and the zoonotic potential of animal infection is the subject of much research. For a long time it was considered that

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Giardia species were host-specific; this resulted in the description of more than 40 species based on the host species in which they were found. This assumption is not universally accepted, however, and current thought is that there are just three structural types or species (Figure 51–4A).

The species affecting man and the majority of domestic animals is referred to as *Giardia duodenalis* and the names *G. intestinalis*, *G. lamblia*, and *Lamblia intestinalis* are synonymous. The other morphologically different species are *G. muris* identified in rodents, birds, and reptiles and *G. agilis* from amphibians.

Biochemical and immunological differences in *Giardia* isolated from humans suggest that there is a great deal of heterogeneity within these strains, while at the same time relatively close relationships exist between certain human and animal strains.[256]

Life Cycle. *Giardia* are flagellate protozoans normally found adherent to epithelial surfaces of the small intestine, especially the middle to lower areas of the villi. The life cycle is simple and direct, the trophozoite stage (motile forms) dividing by binary fission to produce further trophozoites. Species variation exists in the size of the trophozoite and in the shape and size of the median bodies (Figure 51–4A). Intermittently, trophozoites form resistant cyst stages that pass out in the feces of the host.[3] The life cycle is shown in Figure 51–5.

Prevalence in Man and Animals. Infection with *Giardia intestinalis* has been increasingly recognized over the last decade and has been reported as the most common intestinal protozoan parasite in the world in man.[269] Very high prevalence rates of between 20% and 60% are common, particularly in children in developing countries.[28,79,82,107,128,165,171,172,175]

The existence of *Giardia* in domestic animals has been known for many years, but during that time little information has become available on prevalence, pathogenicity, and the disease caused by these parasites in their hosts. *Giardia* has been reported in cattle, sheep, goats, horses, dogs, cats, rodents, and psittacines. Based on limited investigations that involved screening for fecal cysts, the incidence of these parasites varies but can be assumed to be higher in some species than has been reported. Studies in Canada[34] indicate that 18% of sheep and 10% of cattle were infected. In a study conducted in the US, a 100% infection rate was found in young diarrheic calves on two farms.[285] In the UK, nearly 70% of a flock of lambs were infected with *Giardia*, although the presence of the organism was not necessarily associated with clinical signs of disease.[251] Recent studies in cattle described high levels (15% to 32%) of natural prevalence, with the highest level during the winter months.[221] A survey of dogs attending a charity animal hospital in London[247] showed an overall prevalence of infection of 15% with the highest prevalence (30%) in dogs less than 12 months of age. Studies conducted in the US found between 12%[145] and in the US and Canada 1% to 68% of dogs to be infected with *Giardia*.[121] In aviary birds, *Giardia* is frequently encountered in psittacines such as cockatiels, budgerigars, and lovebirds causing enteropathy, weight loss, and death.[97]

Epidemiology and Transmission. Transmission of the dormant, environmentally resistant cyst stage is via the fecal-oral route through contaminated water, contaminated food, or person-to-person, animal-to-animal contact. Cysts can remain infectious for several months under moist and cool conditions, but lose infectivity under dry and hot conditions.

Infection with *Giardia intestinalis* generally is associated with conditions of poor hygiene and sanitation, including poor control of water quality.[15,86,166,209,269] Infection is common among children who attend day-care centers but is also associated with travel.[15,26,99,138,269]

Reports of foodborne transmission of *Giardia* have been made only occasionally. Cysts have been detected in salad, fruit, and other food items.[15] Outbreaks of giardiasis have occurred following the consumption of salmon and cream cheese dip,[191] noodle salad,[197] sandwiches,[278] and fruit salad.[204] Waterborne outbreaks have been reported in the US and the UK.[133] In the former, *Giardia* has been identified more often than any other pathogen in waterborne outbreaks that result in illness. From 1971 to 1985, *Giardia* was identified in 92 of 502 reported waterborne outbreaks, resulting in 24,000 cases of illness.[55] Most cases result from consumption of water intended for drinking or for domestic purposes as well as from consumption from other nonpotable sources by swimmers, divers, hikers, and campers. Human infection in the US has been reported from drinking water contaminated with *Giardia* thought to have originated from beavers.[81]

Because *Giardia* is so widespread among domestic and wild-animal hosts, its zoonotic potential has aroused interest and concern. The occurrence of direct animal-to-human transmission of giardiasis remains controversial. The close association between

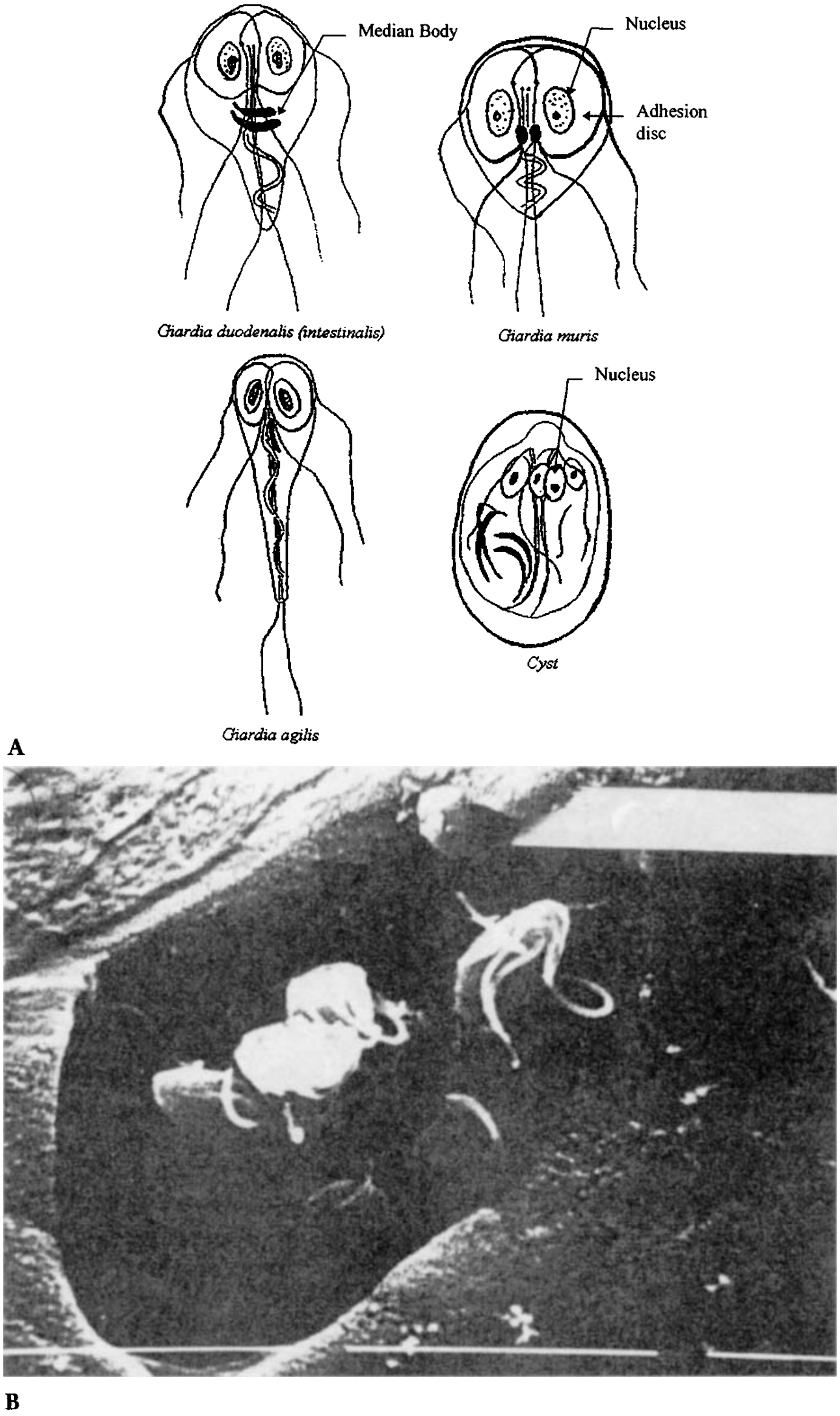

Figure 51–4 Trophozoites of *Giardia*. **A**, Three morphologically distinct species of *Giardia* are recognized and distinguished by the shape of the median body (labeled). In *Giardia duodenalis*, the species infecting domestic mammals including man, the median bodies are in the shape of a "claw hammer." *Source:* Reprinted with permission from M.A. Taylor, Waterborne Parasites: A Veterinary Perspective, *Health and Hygiene*, Vol. 14, p. 56, © 1993, Royal Institute of Public Health and Hygiene. **B**, scanning electron micrograph of trophozoites of *Giardia intestinalis* (small intestine lamb). *Source:* Copyright © 1999, Ministry of Agriculture, Fisheries and Food, U.K.

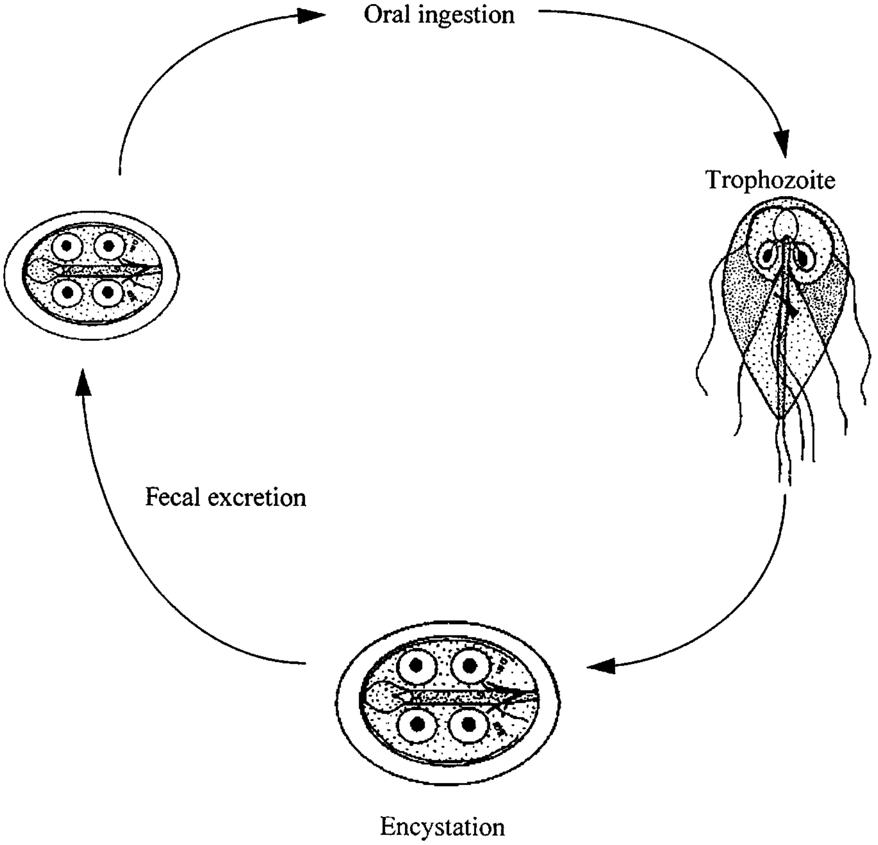

Figure 51–5 Life Cycle of *Giardia intestinalis (duodenalis)*. Trophozoites of *Giardia* are normally found adherent to the epithelial surface of the small intestine where they reproduce by binary fission to produce further trophozoites. Intermittently, trophozoites encyst and are passed out in the feces until ingested by a new host.

humans and companion animals provides opportunity for transmission but unequivocal evidence is lacking. Although anecdotal information indicates that dogs, especially puppies, could serve as sources of human infection, there is no documented evidence of dog-to-human transmission.[121] Cats also might be a source of human infection, but reported cases of feline giardiasis are rare and sometimes circumstantial.[121] Based on reports of giardiasis in hikers and campers who obtained drinking water from untreated surface waters in areas in which few humans are present, wild animals have been suspect reservoir hosts. Aquatic mammals (beavers and muskrats) as well as such aquatic birds as egrets and green herons have been found infected with *Giardia*.[121]

In animals, limited epidemiological studies suggest that direct animal-to-animal contact and fecal soiling is the most likely method of transmission, although water is also likely. Other wild animals may act as reservoirs of infection. Infection in a calf has been established with a human isolate.[221] The role of farm animals in the overall epidemiology of human giardiasis has yet to be investigated.

Clinical Signs. The most-prominent initial signs of giardiasis in man include nausea, inappetence, discomfort in the upper intestine, and fatigue, followed by bursts of foul-smelling watery diarrhea, flatulence, and abdominal distention, usually lasting only a few days.[173,279] Occasionally, this acute stage can last for months, causing malabsorption, weight loss, and debilitation. Chronic infection is marked by recurrent brief episodes or, to a lesser extent, persistent episodes of foul-smelling loose feces, flatulence, and abdominal distention. Lactose intolerance, common during active infection, may persist for a period thereafter. Mortality has seldom been reported.

Giardia infections in many species of animals are often asymptomatic. When disease does occur, the signs often include chronic, pasty diarrhea, weight loss, lethargy, and failure to thrive. The diarrhea may be continuous or intermittent.

Methods of Detection. Diagnosis is based on identification of cysts or trophozoites in feces, or trophozoites in duodenal aspirates or biopsies. Most diagnoses can be made by fecal examination. *Giardia* cysts can be detected in feces by a number of methods. Traditional methods of identification of giardiasis involve direct examination of fecal smears or fecal concentration by formalin-ethyl acetate or zinc sulphate methods and subsequent microscopic examination.[3] It is generally recommended that three consecutive samples are examined as cysts are excreted intermittently,[144] and a single sample identifies only 50% to 75% of positive cases.[3,124] Recent developments include the use of fluorogenic dyes such as fluorescein diacetate, which is taken up by viable cysts, and propidium iodide, which is taken up by nonviable cysts.[238]

Several immunoassays are available commercially. These involve either the identification of cysts by IF[245] or detection of *Giardia* specific antigen by ELISA.[111,181,262]

The use of PCR to amplify target DNA has produced a sensitive and specific assay for *Giardia* based on the detection of the giardin gene (*Giardia*-specific genomic DNA).[35] In addition, the PCR technique is reported to be able to distinguish between pathogenic and nonpathogenic *Giardia* strains[160] and between live and dead cysts by measuring PCR product from giardin mRNA.[161]

Prevention and Control. Control of giardiasis is based on good personal hygiene, proper sanitation, and treatment of drinking water. Because virtually all surface waters can potentially be exposed to *Giardia* contamination from human or animal sources, all *Giardia* cysts should be regarded as potentially infective for humans. Slow sand filtration, filtration with diatomaccous earth, or coagulation-filtration are the methods most commonly used by the water supply industries and usually remove 99% or more of the *Giardia* cysts.[131] Chemical disinfectants such as chlorine, chlorine dioxide, chloramine, and ozone can further reduce the number of infectious cysts in drinking water,[131] but their effectiveness may be reduced by changes in pH, water temperature, turbidity, concentration of disinfectant, and contact time. A combination of filtration and disinfection is often used to best effect.

Conclusion. As with cryptosporidiosis, foodborne infections with *Giardia* are relatively uncommon and the main risk of transmission to man appears to be through foreign travel, especially to underdeveloped countries, or via contamination of drinking water. Implementation of water monitoring and improved treatment facilities should help reduce the risks of waterborne transmission. However, current techniques for the species identification, enumeration, and viability assessment of waterborne cysts are imprecise, which makes the interpretation of the public health significance of detected cysts difficult.

51.4 AMOEBOID PROTOZOA (SARCODINA)

51.4.1 *Entamoeba*

History. *Entamoeba histolytica* is one of six parasitic amoebae of the genus *Entamoeba* known to infect humans.[29] It has also been recorded in cattle, pigs, dogs, cats, and monkeys.[153] In man, the primary route of transmission is from human to human by the fecal-oral route, usually associated with poor hygiene or poor water quality. Infections with *E. histolytica* occur worldwide but are more prevalent in the tropics. There are two morphologically identical forms of *E. histolytica,* one nonpathogenic and the other pathogenic.[29]

Life Cycle. Trophozoites in the intestine, which vary between 12 μm to 60 μm in diameter, divide by binary fission. Transmission occurs when the trophozoites round up, become smaller, and form a cyst (8.5 μm to 19 μm). Nonpathogenic forms of the organism normally live in the lumen of the large intestine. Pathogenic forms invade the mucosa, causing ulceration and dysentery. From there they may be carried via the portal system to the liver and other organs where large abscesses may form (see Figure 51–6).

Prevalence in Man and Animals. It has been estimated that approximately 480 million people, or 12% of the world's population, are infected and that annual mortality is 40,000 to 110,000 people.[115] About 10% of those infected every year have clinical symptoms.[265] Five percent of the US population were estimated to be infected, but its prevalence appeared to be declining.[159]

The incidence in animals is unknown. *Entamoeba histolytica* has been found in naturally infected monkeys of many species throughout the world. Infection in dogs has only been reported sporadically and often through human contacts. Natural infections are apparently rare in pigs and have to be differ-

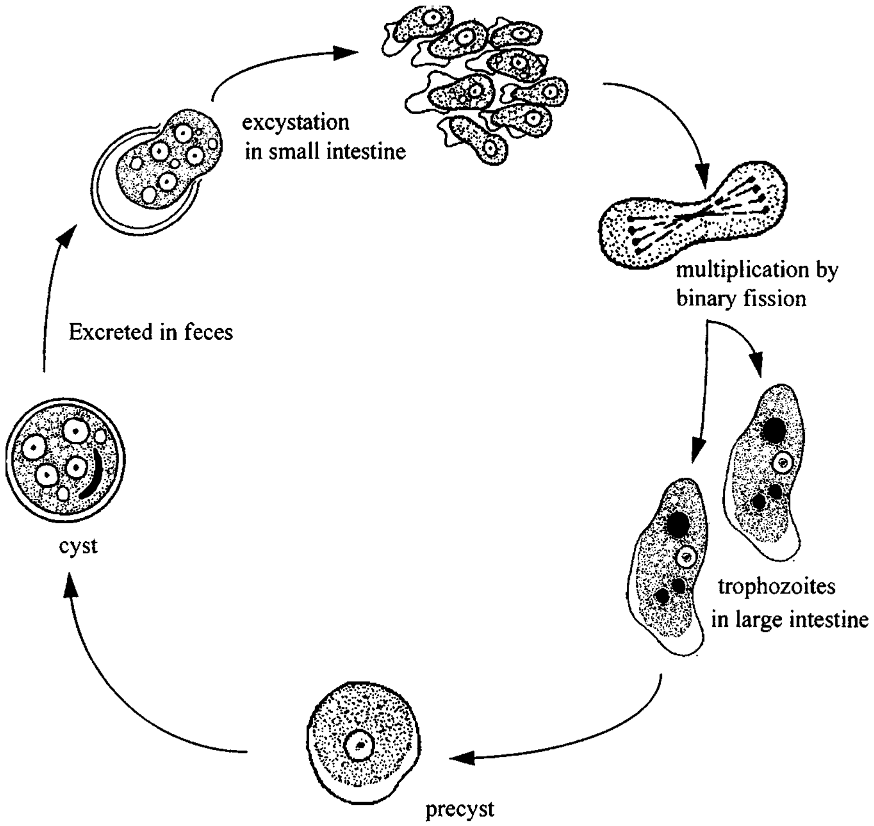

Figure 51–6 Life Cycle of *Entamoeba histolytica.* Trophozoites in the large intestine divide by binary fission to produce further trophozoites. Some trophozoites round up, become smaller, and form cysts, each containing 4 small amoebae, which are passed out in the feces. Once ingested by a new host, excystation occurs in the small intestine releasing the young amoebae, which divide so that each cyst produces 8 small amoebae. *Source:* Adapted from an Atlas of Protozoan Parasites in Animal Tissues, *Agriculture Handbook No. 65,* p. 10, U.S. Department of Agriculture.

entiated from other species that occur in the pig, notably *Entamoeba suis* (*E. polecki*) and *Iodamoeba buetschlii.*[153]

Epidemiology and Transmission. Ingestion of food and drink contaminated with *E. histolytica* cysts from human feces and direct fecal-oral contact are the most common means of infection. Transmission from human to human by the fecal-oral route is usually associated with poor hygiene or poor water quality. Cysts can be introduced into the mouth by soiled hands of food handlers, family members, hospital personnel, and other close personal contacts, by food contamination via flies, and by water contaminated with sewage. Transmission of *E. histolytica* by water is common in third-world countries where much of the drinking water is untreated. The use of human feces for fertilizer is also an important source of infection.[29] Recognized high-risk groups include travelers, immigrants, migrant workers, and immunocompromised individuals and male homosexuals. *E. histolytica* has been found in as many as 32% of homosexual men in North America.[6] Almost all of the strains of *E. histolytica* isolated from AIDS patients have been classified as nonpathogenic.[6,32,108.,276]

Despite documentation of naturally infected nonhuman primates and experimental infection of such other animals as cats and dogs with *E. histolytica,* human infection from any animal source has not been documented. However, knowledge that *E. histolytica* can infect animal hosts serves as warning that, under the right conditions, animals might be a source of *E. histolytica* infecting humans.

Clinical Signs. Most human infections are asymptomatic, with the parasite acting as a harmless commensal organism in as many as 90% of those infected.[61,115] Many asymptomatic individuals never become symptomatic, and usually excrete cysts in the feces for only a short period of time. About 10% of individuals infected with *Entamoeba* *histolytica* have clinical symptoms associated with tropical dysentery with bloody or mucoid diarrhea. Symptoms can range from mild bowel discomfort to diarrhea or dysentery with or without blood and mucus. Infections can last from a few days to several months. The symptoms associated with invasive intestinal amoebiasis are usually nonspecific and can produce ulcerative lesions in such extraintestinal sites as the liver and the skin or even the brain. Amoebic colitis is associated with ulceration of the colon.[47] If ulceration with perforation occurs, the cecum is the area normally involved.[135]

Infections in animals are generally asymptomatic although liver infections may occur in some species of monkey.

Methods of Detection. Trophozoites of *E. histolytica* have a spherical or oval shape (12 μm to 60 μm in diameter) with a large, single nucleus that contains a central karyosome (intranuclear body) and minute, irregularly shaped masses of peripheral chromatin. Cytoplasm generally contains vacuoles and sometimes extends as irregular fingerlike projections (pseudopods) from the margin. Mature cysts often have four, rarely eight, nuclei. Trophozoites and cysts can be stained with iodine in wet mount preparations or with PAS, trichrome, or iron hematoxylin. The organisms can also be cultured in a number of media including Boeck and Drbohlav's, Dobell and Laidlaw's, TYI-S-33,[64] and Robinson's medium.[63] Serological tests that have been evaluated for the diagnosis of *E. histolytica* infections include ELISA,[186] latex agglutination, complement fixation;[158] and indirect hemagglutination.[23] A number of PCR methods have also been used to detect *E. histolytica* in clinical samples.[2,49,219,248] Isoenzyme markers can be used to differentiate the two forms, but there is some debate as to whether the two types represent different species or if they can change from one type to another under certain circumstances.[29]

Prevention and Control. Prevention is by elimination of fecal contamination of food and water. Good personal hygiene, especially thorough washing of hands by food handlers, good sanitation, and high-quality water treatment are essential for preventing transmission.

Conclusion. Although waterborne transmission of *Entamoeba histolytica* occurs throughout third-world countries, and the prevalence of infection remains relatively high, the incidence in industrialized societies appears to be decreasing. The main risk of infection is therefore from drinking untreated and fecally contaminated water.

51.5 BLASTOCYSTA

51.5.1 *Blastocystis*

History. *Blastocystis* was described for many years as a yeast but is now considered to be a protozoan in the subphylum Blastocysta.[134] The organism is found in the intestinal tract of man and in many animals including monkeys, pigs, birds, rodents, snakes, and invertebrates. Infection generally is asymptomatic but it is becoming increasingly associated with gastrointestinal disease in man.[290]

Life Cycle. The life cycle first described for this organism was complex, involving binary fission of a binucleate stage followed by autogamy (sexual reproduction) to form primary cysts. These cysts were thought to produce spores by multiple budding leading to the production of resistant secondary cysts. In this life cycle, asexual merogony and sporogony also were described.[5] It was proposed later that only asexual reproduction occurred, with division occurring by at least four different modes described as binary fission, plasmotomy, endodyogeny, and merogony.[290] Further studies involving electron microscopy have failed to reveal endodyogeny, merogony, or sporogony. Binary fission is therefore the only proven method of reproduction at the present time.[24] The predominent form present in the human colon is a small nonvacuolated cell. As the nonvacuolated form passes through the colon, a multivacuolar form develops, which leads to the ultimate formation of a cyst that is passed out in the feces (Figure 51–7).

Prevalence in Man and Animals. Reports of the prevalence of *Blastocystis* are very variable. Studies in man show varying levels of infection with *B. hominis* in both healthy patients and patients with diarrhea.[148,164,185] Infection appears to be more common in adults than children.[66,142,206]

Blastocystis has been reported in a wide range of animals. In mammals it has been reported in a pig-tailed macaque,[167] monkeys,[286] and pigs.[33,192] The pathogenicity of *Blastocystis* in pigs is uncertain. Cross-transmission studies have indicated a low host specificity for *Blastocystis*,[192] and it is likely that the parasite will be found in other farm livestock.

The organism has also been reported in chickens,[18,286] ducks,[193] partridge and pheasants,[252] and reptiles.[255]

Epidemiology and Transmission. It is generally presumed that *B. hominis* is transmitted by the fe-

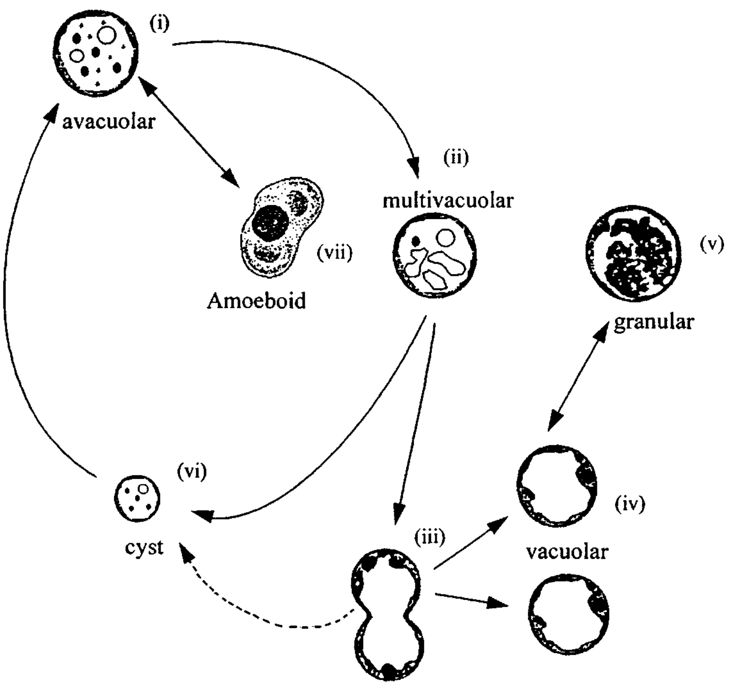

Figure 51–7 Life Cycle of *Blastocystis hominis.* Various life cycles have been described for *Blastocystis hominis;* the following description is that proposed by Boreham and Stenzel. Small nonvacuolated cells (i) present in the colon develop into multivacuolar forms (ii), which can then develop into resistant cyst stages (vi) and pass out in the feces. The typical vacuolar form (iii) arises from the multivacuolar form by coalescence of the smaller vacuoles and divides by binary fission. Under adverse conditions, the vacuolar form may transform to the granular form (v) or may develop into a cyst. It is thought that an amoeboid form (vii) may, on occasions, arise from the avacuolar form. Infection of a new host probably occurs following ingestion of the cyst form, although it may be that the vacuolar form, which appears in feces, may also be infective. *Source:* Adapted with permission from P.F.L. Boreham and D.J. Stenzel, *Blastocystis in Humans and Animals: Morphology, Biology, and Epizootiology,* Advances in Parasitology, Vol. 32, p. 40, © 1993, Academic Press, Inc.

cal-oral route in a similar manner to other enteric protozoa.[101] Familial transmission[114] and transmission within institutes for the mentally retarded[287] have been reported. Waterborne and foodborne transmission are possible routes of infection[101] but have not been investigated. Infections in man have been linked with foreign travel.[66,142,233] In one study, 44% of patients infected with *B. hominis* had a history of exposure to pets or farm animals.[66]

Clinical Signs. It is still unclear whether *Blastocystis* is a pathogen or commensal. Symptoms usually associated with human infection with *B. hominis* include diarrhea, abdominal discomfort, anorexia, flatulence, and other nonspecific gastrointestinal effects. It may be the case that the organism is pathogenic only under specific conditions such as immunosuppression, poor nutrition, or concurrent infections.[24]

Studies in several species of animals suggest that *Blastocystis* is not pathogenic to the host. Large numbers of *Blastocystis* were present in diarrheic pigs sampled from four farms in the UK but no evidence was found to suggest that *Blastocystis* had a pathogenic role.[33] Similar observations were made in partridges in the UK.[252] Both studies suggested that diarrhea may produce conditions suitable for growth of *Blastocystis* rather than the reverse.

Methods of Detection. In fecal samples, *B. hominis* is brightly refractile, of widely variable diameter (4 µm to 15 µm), with a thin band of peripheral cytoplasm surrounding a central vacuole. In severe infections, an amoeboid form may be seen.[290] Diagnosis is based on microscopic identification of characteristic refractile, pleomorphic forms either using bright field optics or by differential interface contrast optics. Fecal smears can be heat fixed or gluteraldehyde fixed and stained with Giemsa, trichrome, or iron hematoxylin. The organism can be cultured successfully in Boeck Drbohlav's, Dobell and Laidlaw's medium, or Robinson's medium.[252] Limited immunological methods of diagnosis have been developed. An immunofluorescent staining method using rabbit antiserum raised to whole *B. hominis* antigen has been described.[290]

Prevention and Control. Prevention is as with other intestinal protozoa—through good hygiene, especially thorough washing of hands by food handlers, and good sanitation aimed at preventing transmission.

Conclusion. *Blastocystis* is increasingly reported as an intestinal infection of man, but it is not clear whether it acts as a pathogen. Transmission is by the fecal-oral route and waterborne and foodborne transmission are potential routes of infection, but have not as yet been investigated to any great extent.

51.6 CILATED PROTOZOA (CILOPHORA)

51.6.1 *Balantidium*

History. *Balantidium coli* occurs in man, other primates, pigs, and occasionally in the dog, rat, and ruminants. The pig appears to be the primary host, and in it *B. coli* generally is regarded as a commensal of the large intestine where it lives on starch, ingesta, and bacteria.[153] It has only rarely been reported as a foodborne or waterborne zoonotic.

Life Cycle. Trophozoites in the lumen or tissues multiply by binary fission and under certain conditions transform into cysts that pass from the body in the feces (Figure 51–8A).

Prevalence in Man and Animals. The organism is found worldwide in humans, but the prevalence of disease based primarily on case reports appears low. Only 722 cases were reported worldwide by 1960 and few have been reported since then. The low prevalence may be in part a combination of lack of recognition of the organism and inappropriate diagnostic procedures.

Epidemiology and Transmission. Transmission via the fecal-oral route is from human to human and possibly from pigs or rats to humans. Transmission is facilitated in confined environments in which hygiene is poor. The cyst form is the source of infection and cysts can remain viable for days or weeks in moist pig feces.

Flies are possible mechanical vectors. Although pigs have been implicated as a major source of human infection, their role is still a source of controversy. In a review of the literature, more than 50% of the persons with balantidiasis reported contact with pigs.[268] In one case, a family claimed that infection followed ingestion of raw pork sausage. Surveys of fecal samples collected throughout New Guinea revealed prevalence rates of infection with *Balantidium* as high as 29% in some areas in which people lived in intimate contact with pigs. A waterborne outbreak implicating pigs as the source of infection has been reported.[267]

Balantidium infection in monkeys and primates is normally an endemic infection maintained by the animals themselves.

Clinical Signs. In man, *B. coli* is a widespread asymptomatic commensal but may on occasions produce superficial to deep ulcers that result in intermittent diarrhea or dysentery. Clinical infections resemble amoebiasis with diarrhea, abdominal pain, nausea, vomiting, and tenesmus. Sometimes the diarrhea contains mucus, blood, or pus. Chronic infections are characterized by alternate episodes of diarrhea and constipation with cramps, abdominal pain, and tenesmus. Rare complications include perforation of the bowel wall and extraintestinal infection of the liver, vagina, ureter, and bladder.

B. coli is also a widespread asymptomatic commensal in the intestine of pigs, where it may invade the mucosa and cause ulceration and mild to severe diarrhea.[103] Severe clinical disease also has been reported for nonhuman primates, especially the great apes.

Methods of Detection. *Balantidium* are large, highly motile ciliated protozoa and can be readily identified in wet mount preparations stained with iodine in which they appear orange with black starch granules (Figure 51–8B). The trophozoites are oval with a slightly pointed anterior end, have a thin membrane covered with cilia, have foamy cytoplasm with a large macronucleus and a tiny micronucleus, and measure about 40 μm to 80 μm by 25 μm to 45 μm. Cysts are round to oval, are surrounded by a thick wall, contain a macro- and micronucleus, and measure about 40 μm to 65 μm.

In fresh fecal smears, the organism can be stained with trichrome or iron hematoxylin. Cultural isolation is not usually necessary, although the organism has been successfully cultured in gastrin mucin media.[146]

Prevention and Control. Prevention is the same as for amoebiasis. Under severe environmental conditions, water thought to be contaminated should be boiled before use.

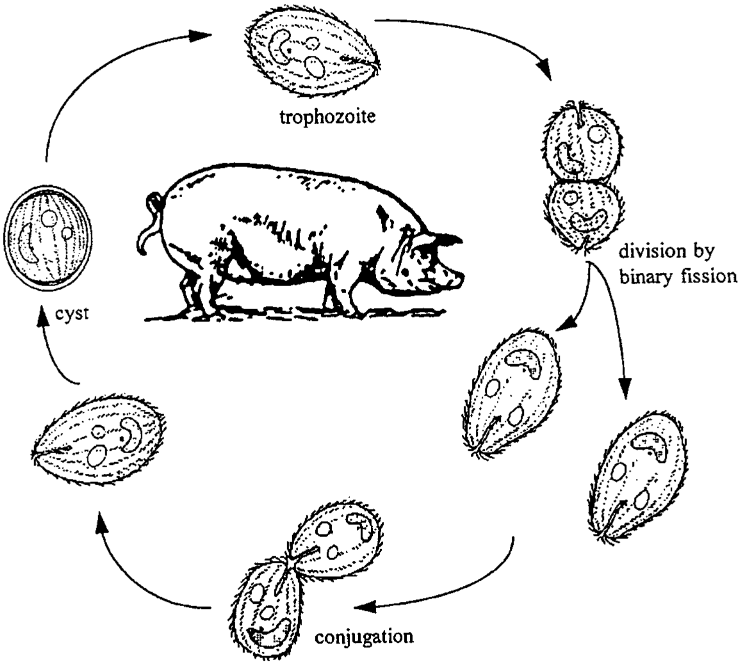

Figure 51–8A Life Cycle of *Balantidium coli.* Trophozoites present in the lumen or tissues of the large intestine divide by binary fission to produce further trophozoites. Conjugation resulting in exchange of genetic material has been observed. Under certain conditions trophozoites transform into cysts and are passed out in the feces.

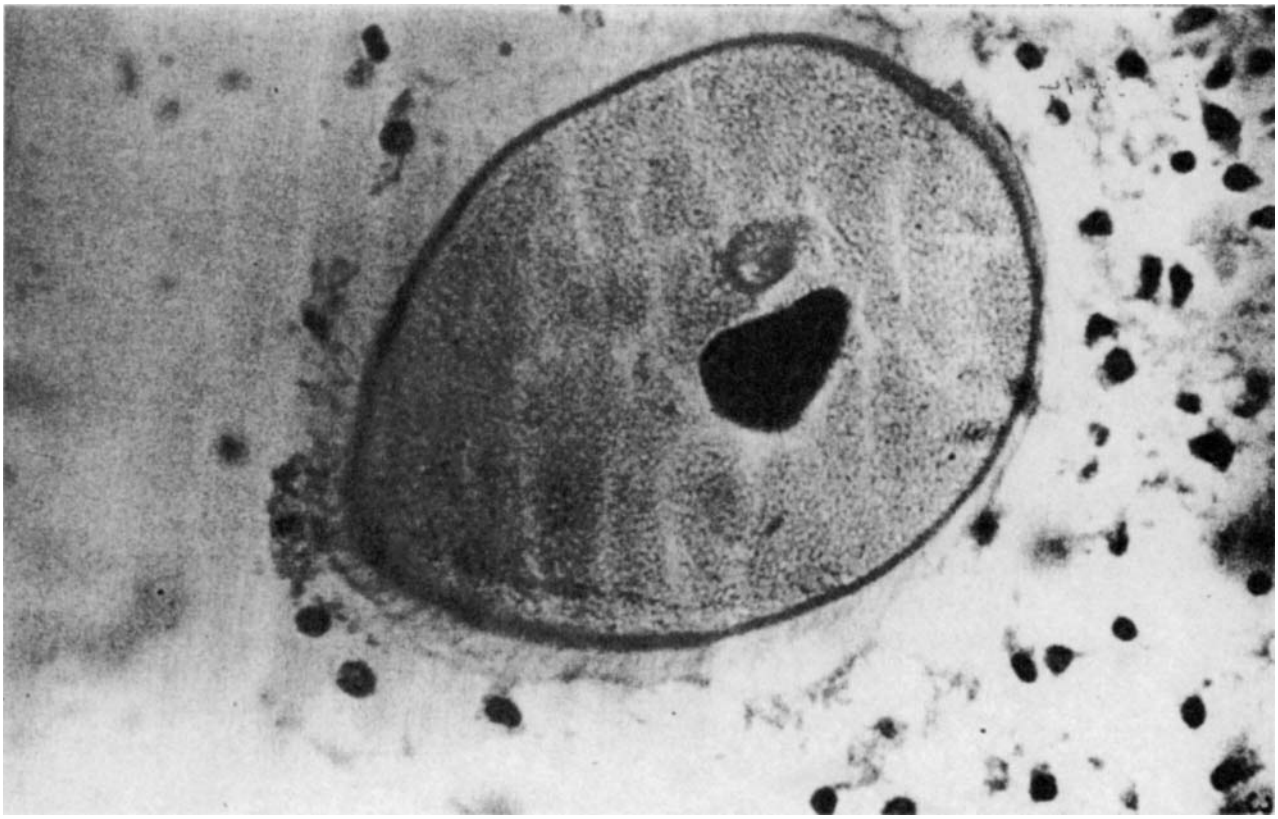

Figure 51–8B Trophozoite of *Balantidium coli* [photographed at x640 Haematoxylin-Eosin stain]. The large macronucleus is clearly visible. *Source:* Copyright © 1999, Ministry of Agriculture, Fisheries and Food, U.K.

Conclusion. *Balantidium* infections in man are relatively infrequent and the epidemiology is not fully understood. Infection is likely to be due to contamination of water or uncooked vegetables. The organism is primarily a parasite of pigs and it is thought that pigs act as a reservoir of infection for man.

51.7 MICROSPORA

51.7.1 Microsporidia

History. "Microsporidia" is a nontaxonomic term used to describe organisms in the phylum Microspora, which contains over 100 genera and 1,000 species that are ubiquitous in nature and infect a wide range of vertebrate and invertebrate hosts. Five genera have been implicated in human disease and infections have been documented from nonmammalian genera, suggesting some lack of host specificity. *Microsporidia* are obligate intracellular parasites that have been found in every major group of animals, and have been reported in man and a wide range of domestic animals and wildlife including reptiles, amphibians, and fish.[39] In man, they are gaining attention as opportunistic pathogens in AIDS patients.[90,147,188,189,207,227,231] At least 5 genera are known to infect and produce disease in man: *Encephalitozoon, Enterocytozoon, Nosema, Pleistophora*, and *Septata*.[30,120]

Life Cycle. The infective stages are resistant spores, which can survive in moist, cool conditions for 1 or more years. Spores consist of an infective agent (sporoplasm) and an extrusion apparatus consisting of a polar tube attached to an anchoring disc (polar sac). When spores are ingested by a new host, the polar tube is everted and attaches to a host cell, and the sporoplasm is inoculated into the host cell cytoplasm. This is followed by a phase of multiple fission (merogony) leading to sporogony resulting in the production of spores (Figures 51–9 and 51–10).

Prevalence in Man and Animals. In humans, microsporidia have been found particularly in patients with immunosuppression such as AIDS patients. Studies from patients with AIDS enteropathy showed 30% to 35% to carry microsporidia.[147,246] The two most common intestinal microsporidia in patients with AIDS are *Enterocytozoon bieneusi* and *Septata intestinalis*.[31] As medical practitioners become aware of microsporidia, they are increasingly reported, although their origin remains uncertain. They may be previously unrecognized natural parasites of man or zoonotic infections from other animals.[39] Serological evidence suggests that latent infections may be quite common in man.[39] In a study to investigate the presence of antibodies to *Nosema cuniculi* in human and animal sera, normal blood donors in Singapore did not have detectable antibody titers, but 6 out of 69 apparently normal Caucasians had a low titer of antibodies.[237] High titers of antibodies were also detected in laboratory rabbits, and significant titers in 38 out of 40 sheep.

Encephalitozoon cuniculi is widespread in rodents, rabbits, carnivores, and nonhuman primates as well as occurring commonly in man.[39] Microsporidia belonging to the genera *Nosema, Glugea, Pleistophora, Haplosporidia, Dermocystidium*, and *Sporozoon* commonly infect lower vertebrates (fish, amphibians) and have occasionally been reported in reptiles.[212] There are numerous species in fish, some of which are responsible for mortality or pathological abnormalities sufficient to make affected fish unmarketable.[38]

Epidemiology and Transmission. The widespread occurrence of intestinal microsporidial infection in patients with HIV and the potential for human-to-human fecal-oral transmission[232] suggest that microsporidia may be common enteric human pathogens worldwide, and their frequency may be related to the adequacy of sanitation in the environment. In general, infections appear to be more common in developing countries, but expanding poverty, urban migration, air travel, and immigrant populations in conjunction with other social and environmental changes may explain some of the increase in more developed countries.

The main methods of infection in man largely are unknown, but contaminated water has been suspected.[272] Human-to-human transmission has been postulated to occur via the fecal-oral route, urinary-oral route, eye infection, sexual transmission, or transplacentally.[163]

Only a few species of microsporidia have been recorded from mammals, although one, *Encephalitozoon cuniculi*, has a wide geographic distribution and is transmitted orally and transplacentally.[38] Other microsporidia in mammals may simply be opportunistic parasites causing illness under conditions of immunoincompetence. The presence of large numbers of species of microsporidia in fish and amphibians suggests that the aqueous environment may be a significant factor in their transmission.

Clinical Signs. The clinical conditions caused by microsporidia vary widely; some cause gastrointestinal disturbance, others cause systemic disease, ne-

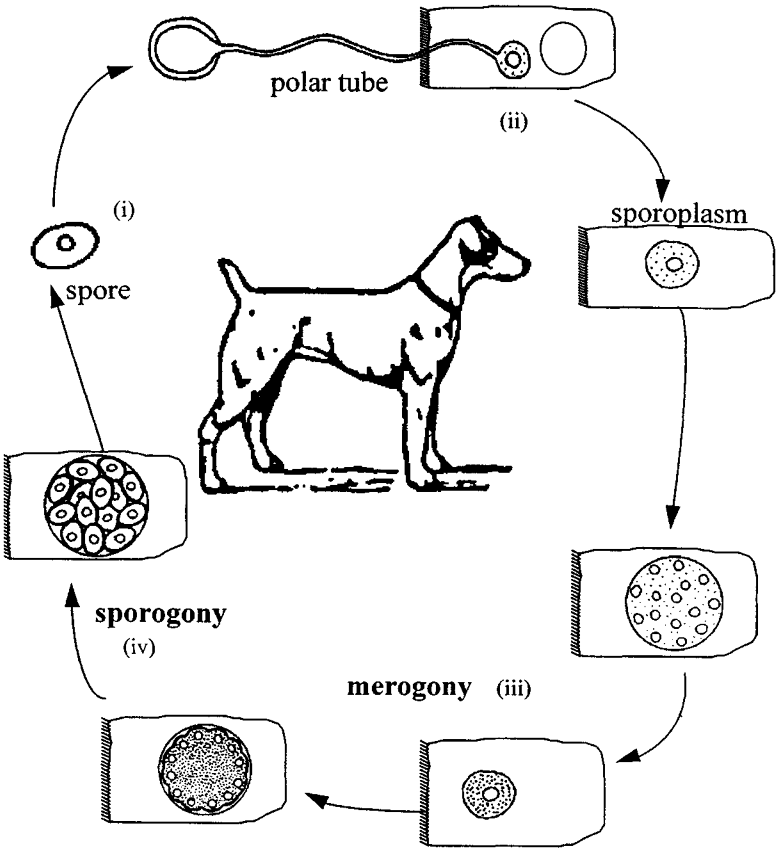

Figure 51–9 Life Cycle of Microsporidia (*Encephalitozoon cuniculi*). Ingestion of a spore (i) by a new host leads to the extrusion of a polar tube, which attaches to a host cell by an anchoring disc (polar sac), and inoculation of the sporoplasm into the cytoplasm of the host cell (ii). There then follows a period of merogony (iii), followed by sporogony (iv), leading to the production of spores.

phritis, and ascites, while others affect the nervous system. Chronic diarrhea, dehydration, and weight loss are the most common signs of microsporidiosis. Most infections are found in HIV patients, with prolonged diarrhea lasting up to 4 years reported and mortality rates of more than 50%.[30,207] Abdominal pain, vomiting, and fever appear to accompany biliary infections. Reported infections in man include keratitis, conjunctivitis, hepatitis, peritonitis, myositis, central nervous system infection, renal disease, and sinusitis.[37,40,176,203,207,227]

E. cuniculi can cause a variety of neurological syndromes in infected mammals. In dogs, transplacental infection of puppies can lead to temperamental changes similar to rabies infection or signs similar to distemper virus leading to fading puppy syndrome. Rabbits also can become infected and often exhibit severe neurological disorders.[39] A plethora of species exist in lower vertebrates, particularly fish, causing a diverse range of clinical and pathological signs affecting the skin, muscle, nervous tissue, intestine, and gonads.[212]

Methods of Detection. The microsporidia are very tiny protozoa between 1 μm and 5 μm in size. Staining techniques include a modified Trichrome staining method,[223] a modification of the standard one-step Trichrome stain using a 10-fold concentration of chromotrope 2R[104] and a rapid fluorescence technique using Uvitex 2B.[263] Although acid-fast, they are difficult to detect and identify by light microscopy, and identification is frequently based on electron microscopy. *Encephalitozoan cuniculi* has been cultured in monolayer culture of rabbit choroid

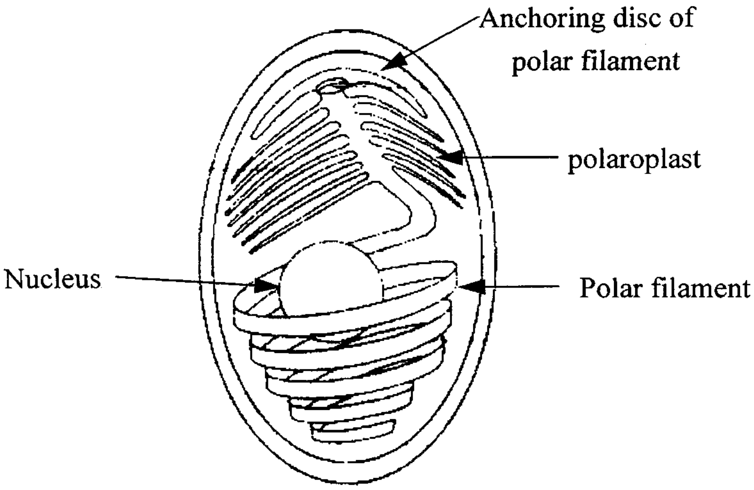

Figure 51–10 Diagrammatic Representation of a Microsporidian Spore Showing the Internal Structures Notably the Extrusion Apparatus Comprising the Coiled Polar Tube and Anchoring Disc. When spores are ingested by a new host, the polar tube is everted. When fully extended, and if the tip of the tube penetrates a host cell, the sporoplasm (nucleus and cytoplasm) passes through the tube and is inoculated into the cytoplasm of the host cell.

plexus cells and numerous cell lines will support its growth. Madin Derby Canine Kidney (MDCK) cells are particularly successful.[41] A method capable of detecting culture-derived *E. cuniculi,* and *E. hellem* and two other human microsporidia, *Enterocytozoon bienusi* and *Septata intestinalis* in formalin-fixed fecal and urine samples, respectively, has been described.[4] An ELISA for detection of antibodies to *E. cuniculi* uses antigen produced from spores derived from tissue culture.[39]

Prevention and Control. Infection is seen predominantly in immunocompromised individuals, and recommendations made for other opportunistic protozoan infections should be followed.

Conclusion. Microsporidia increasingly are being recognized as important human intestinal pathogens mainly in patients infected with HIV but many questions relating to the epidemiology, routes of transmission, and diagnosis remain unanswered. Although many aquatic animals are hosts to microsporidian species, it is not known whether the microsporidia that produce disease in humans have reservoir animal hosts. Microsporidia can survive in water for relatively long periods of time as resistant spores, and because they are much smaller than the resistant stages of other protozoan parasites, they are much more likely to pass through filters used in water treatment processes and to contaminate drinking water.

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52 Foodborne Viruses

E. Owen Caul

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52.1 INTRODUCTION

Human fecal viruses are significant causes of foodborne illness, although it is not possible, at present, to quantify the extent of the problem on a global scale. This is largely due to the lack of comprehensive surveillance systems allied to laboratory diagnosis in most parts of the world. It is, however, clear that enteric viruses commonly are recognized as a cause of human disease associated with foodborne transmission and are almost certainly under-ascertained on a worldwide basis. The diseases caused by foodborne human enteric viruses fall into two major distinct clinical entities that present as viral gastroenteritis or viral hepatitis. Foodborne viral gastroenteritis increasingly is recognized and appears to be a significant public health hazard while foodborne viral hepatitis is less common, at least in the developed world. To understand the mechanisms involved in foodborne transmission, it is fundamental to consider the natural history of these two distinct clinical syndromes, which underpins why foodborne outbreaks occur and has lessons for prevention and management.

As a result of the extensive published epidemiological and laboratory investigations of outbreaks of nonbacterial gastroenteritis, it is apparent that the small round structured viruses (SRSVs) or Norwalk/Norwalk-like viruses commonly are implicated in foodborne gastroenteritis worldwide. In the United Kingdom (UK), SRSVs represent the most significant viral problem to the food industry. While astroviruses and caliciviruses represent a minority of foodborne outbreaks, all three will now be considered. Other enteric diarrhea viruses (eg, Adenovirus types 40/41) have either not been implicated in foodborne gastroenteritis or are exceptionally rare (eg, rotavirus). It is noteworthy that while Group A rotaviruses are recognized as the most common and important cause of childhood diarrhea worldwide, they rarely have been incriminated in foodborne viral gastroenteritis. Epidemiological studies have demonstrated that young children (< 2 yrs old) are particularly susceptible to severe gastroenteritis and that transmission occurs by the fecal/oral route from child to child. Clinically recognizable Group A rotavirus infections in adults are relatively uncommon, probably as a result of immunity derived from infection in childhood and a decreased host susceptibility. Immunity wanes in the elderly and they become susceptible to symptomatic infection with this virus. While Group A rotavirus can survive on fresh fruit and vegetables, transmission to man rarely has been documented. The burden of illness in young children is so large that foodborne transmission, if it occurs, would not be easily recognized. In contrast, Group B rotaviruses have been well recognized as a cause of severe gastroenteritis in adults in China where transmission through drinking water was established. This is unique to China and has not been documented in the UK, Europe, the United States (US), or elsewhere in the world. In view of the paucity of incidents incriminating rotaviruses in foodborne transmission, this virus will not be considered further.

Finally, outbreaks of foodborne viral hepatitis caused by Hepatitis A also are recognized and these will additionally be considered. A résumé of viral gastroenteritis, clinical aspects, pathogenesis, and epidemiology will now be considered followed by Hepatitis A. The laboratory diagnosis of these infections will follow as a separate section and the chapter will conclude with aspects of prevention and control.

52.2 TAXONOMY OF FOODBORNE VIRUSES

The recent molecular characterization of three strains of SRSV has clarified the taxonomic status of this group of viruses.[50,68,113,139] Thus the overall genome organization of SRSVs is compatible with their classification within the Caliciviridiae family[141] and is compatible with early studies on the protein and nucleic acid composition of Norwalk virus.[90,167] SRSVs can be divided into two distinct genetic groups (genotypes I and II) based on the degree of homology recognized within the RNA polymerase or capsid region of the viral genome.[85,86,87,148,225] Importantly, molecular studies also have demonstrated fundamental differences in the genome organization of SRSVs and the classical human enteric caliciviruses.[35,50,140,152,166] Thus SRSVs and the classical human enteric caliciviruses represent two distinct subgroups within the Caliciviridiae family,[50] despite one report suggesting similarities between SRSVs and classical human caliciviruses.[60]

The complete genome organization of astroviruses also has been described recently[228] and these have been allocated to a new family, the Astroviridiae.[171] Hepatitis A has been established as a unique member of the Picornaviridiae and has been classified as a new genus, Hepatovirus.[106] Hepatitis E only recently has been characterized at the molecular level[214] and has been tentatively classified within the family Caliciviridiae. The taxonomic status of all these viruses with their diseases in man is shown in Table 52–1.

52.3 VIRAL GASTROENTERITIS

Diarrheal disease in man, microbiologically distinct from bacterial gastroenteritis, was initially reported more than 50 years ago in the southern US. This clinical syndrome was originally described by Zahorsky[232] as hyperemesis hiemis or winter vomiting disease that reflected both its clinical presentation and distinct seasonality. The illness was documented initially in families where a high secondary attack rate occurred, suggesting a high infectivity. Patients presented with an acute, short, self-limiting episode of vomiting with diarrhea as the main presenting feature. Subsequently, this clinical presentation also was recognized by clinicians in Britain,[27,82] and further laboratory studies established that it was distinguishable from bacterial gastroenteritis. This became particularly clear as a result of investigations of institutional outbreaks where stool cultures were consistently shown to be negative for pathogenic bacteria using the traditional bacteriological techniques available at that time.

Following Zahorsky's original description, other clinicians used their own synonyms to describe the illnesses they investigated; as a result, the literature abounds with a variety of terms. Thus, nonbacterial gastroenteritis, epidemic nonbacterial gastroenteritis, epidemic gastroenteritis, acute infectious nonbacterial gastroenteritis, acute nonbacterial gastroenteritis, and epidemic vomiting disease are examples of the synonyms used between the 1940s and early 1970s to describe this gastrointestinal syndrome. Subsequent studies, using the oral inoculation of human volunteers with a fecal filtrate from symptomatic patients, demonstrated that a transmissible agent was present in human stools.[1,51,70,80,114,198,211] This approach established the potential for a viral cause for these illnesses, and details documenting incubation periods and symptoms produced in volunteers were recorded. Laboratory studies directed toward the isolation of a bacterial pathogen or a viral agent failed, although the natural history of the transmissible agent was comprehensively described.

Table 52–1 Diseases and Taxonomic Status of Foodborne Viruses

Virus	*Disease*	*Family*	*Nucleic Acid*	*Genotypes*	*Serotypes*
SRSV	Epidemic gastroenteritis (all age groups)	Caliciviridae	Single stranded RNA	2	Multiple
Classical Calicivirus	Mainly pediatric gastroenteritis	Caliciviridae	Single stranded RNA	2 (distinct from SRSV)	4
Astrovirus	Mainly pediatric gastroenteritis	Astroviridae	Single stranded RNA	1	7
Hepatitis A	Hepatitis	Hepatovirus	Single stranded RNA	1	1
Hepatitis E	Hepatitis	? Caliciviridae	Single stranded RNA	?	1 serotype currently recognized

Many years elapsed before an investigation of a school outbreak of epidemic gastroenteritis in Norwalk, Ohio, described for the first time a viral etiology for epidemic nonbacterial gastroenteritis.[119] American workers prepared fecal filtrates from stool samples collected from symptomatic patients in this outbreak, which they used to infect human volunteers. They then applied the technique of immune electron microscopy to the examination of stool samples from the volunteers. These studies identified a previously undescribed 27 nm virus-like particle and the Americans coined the term Norwalk virus for this agent in recognition of the geographical setting in which the outbreak occurred. Further detailed studies in human volunteers established the Norwalk virus as the etiological agent responsible for the original outbreak in the primary school.[115] Subsequent laboratory investigations demonstrated either seroconversions or rising IgG titres in serum samples from symptomatic volunteers as well as virus excretion in stool samples. These pioneering human volunteer studies largely fulfilled Koch's postulates and subsequent studies addressed the pathogenesis and clinical features of this new virus. The American workers carried out further volunteer studies using the Norwalk agent and jejunal biopsies were collected from infected volunteers with acute disease. Histopathological studies on these jejunal biopsies provided new insights into the cell tropism of Norwalk virus in the small intestine,[2,206] and all of these studies represented a milestone in expanding our knowledge of human viral gastroenteritis. Further electron microscopical studies on human stools by American workers identified morphologically similar viruses to the Norwalk virus in two separate outbreaks of nonbacterial gastroenteritis. These viruses were called Montgomery County and Hawaii virus respectively,[115] reflecting the geographical setting of the affected patients. The practice of naming small round viruses identified in different outbreaks according to their geographical setting became common[30] and has been continued to the present day. These electron microscopical studies on Norwalk virus heralded the discovery of a variety of morphologically distinct viruses in human stool samples.

The application of electron microscopy to the examination of diarrheic human stool samples became a common investigation worldwide and subsequently resulted in major advances in our understanding of the etiology of nonbacterial gastroenteritis. Rotaviruses were rapidly identified as major pathogens in endemic infantile gastroenteritis, and other taxonomically distinct viruses, including fastidious adenoviruses, were added to the increasing list of newly described enteric diarrhea viruses.[48] Numerous reports subsequently demonstrated an apparently wide range of small round viruses that varied in size and surface morphology. In many studies, small round viruses were identified by immune electron microscopy (IEM) as a result of aggregation following the use of human sera. The addition of human antibody to fecal viruses masks the surface morphology of virus particles, and this resulted in a confusing picture of the etiology of nonbacterial gastroenteritis.[30] In other reports, small round viruses were identified from their classical surface morphology in the electron microscope after negative staining and detailed descriptions of the surface morphology of astroviruses and classical caliciviruses were published for the first time.[160–162]

In an attempt to bring some order to the standardized recognition and reporting of the many small round viruses being described in the literature, an interim classification scheme was published.[36] This scheme was adopted to aid national surveillance within the UK and to establish which of the small round viruses were related causally to viral gastroenteritis. The study defined comparative morphological criteria derived from examining taxonomically established human and animal small round viruses. The authors concluded that small round human enteric viruses could be readily subdivided into those viruses that lacked a resolvable surface structure and that could be described as featureless viruses and those that had an obvious resolvable surface structure in the electron microscope. The first group (featureless viruses) included both picornaviruses (which do not cause gastroenteritis) and parvovirus-like particles that were represented by the Wollan, Ditchling, Cockle, and the Australian Paramatta[49] agents. All four agents (parvovirus-like) previously had been implicated in outbreaks of nonbacterial gastroenteritis but conclusive evidence of their role in human disease never has been proven and this remains the situation to date.[5,7,30]

The second group of small round viruses all possessed a resolvable surface morphology in the electron microscope that often was descriptive and included the previously described and taxonomically distinct astroviruses, and "classical" caliciviruses. Other viruses morphologically distinct from both astroviruses and classical caliciviruses shared a common surface structure and were described as virus particles with an amorphous surface structure lacking geometric symmetry and possessing a ragged outline.[36] This latter group of viruses was represented by the prototype Norwalk virus and subsequently Hawaii, Montgomery County, and many other morphologically indistinguishable viruses were included. This latter subgroup of viruses, which were distinguishable morphologically from both astroviruses and the classical caliciviruses, were called collectively SRSV or Norwalk-like viruses. A prerequisite of this study was that all viruses were examined without the addition of human antibody to ensure that any surface structure would not be obscured. A modification of this interim classification scheme is shown in Figure 52–1 and provides examples of those currently recognized viruses that are classified as SRSVs. This interim classification scheme has proved reliable and reproducible in accurately identifying SRSVs and recently has been adopted by other workers.[115]

An important aspect of this classification scheme was that it recognized fundamental morphological differences between SRSVs and the classical caliciviruses. These observations also were supported by both epidemiological and immunological distinctions[18,30] discussed later. Recent advances in the molecular characterization of SRSVs and the classical caliciviruses also have demonstrated significant differences at the molecular level[139,140] despite the recent classification of SRSVs within the family Caliciviridiae.[68,113,139] Thus, the initial distinctions made at the epidemiological, immunobiological, and electron microscopical level have been confirmed at the molecular level. These results suggest that SRSVs are a distinct subgroup within the Caliciviridiae family and are distinguishable from the classical human caliciviruses; thus SRSVs and the classical caliciviruses will be considered separately, which emphasizes their different epidemiological roles in human disease and recognizes their distinction at the molecular level.

52.3.1 Clinical Aspects of Viral Gastroenteritis

SRSV. Detailed information on the clinical manifestations of SRSVs has been established from both epidemiological as well as comprehensive volunteer studies.[115,116,118] Illness in naturally acquired infections, as well as those induced in volunteers, produced by Norwalk virus, Hawaii agent, the Montgomery County agent, and the Snow Mountain agent and other SRSVs, is clinically indistinguishable.[73,115,231] Volunteer studies and epidemiological studies have documented a mean incubation period of approximately 24 hours[30,115] with a dose-dependent range of 15 to 50 hours.[33,34] Symptoms are sudden in onset without a defined program and often are accompanied by nausea and vomiting, which can be projectile and severe. A low-grade fever and diarrhea are common presentations, with the latter being relatively mild to moderate in severity. A significant observation, in contrast to bacterial gastroenteritis, was that diarrheal stools did not contain blood, mucus, or white cells. Other symptoms included mild abdominal pain or cramps, malaise, and headaches. Vomiting may arise from a decrease in gastric mobility giving rise to a reflux action into the stomach.[30,34,115,116] Respiratory symptoms have not been documented and volunteer studies, using nasopharyngeal washings from an acutely ill volunteer, did not induce disease on passage to other volunteers.[70]

In general, SRSV infections are mild and self-limited, and affected patients rarely require hospitaliza-

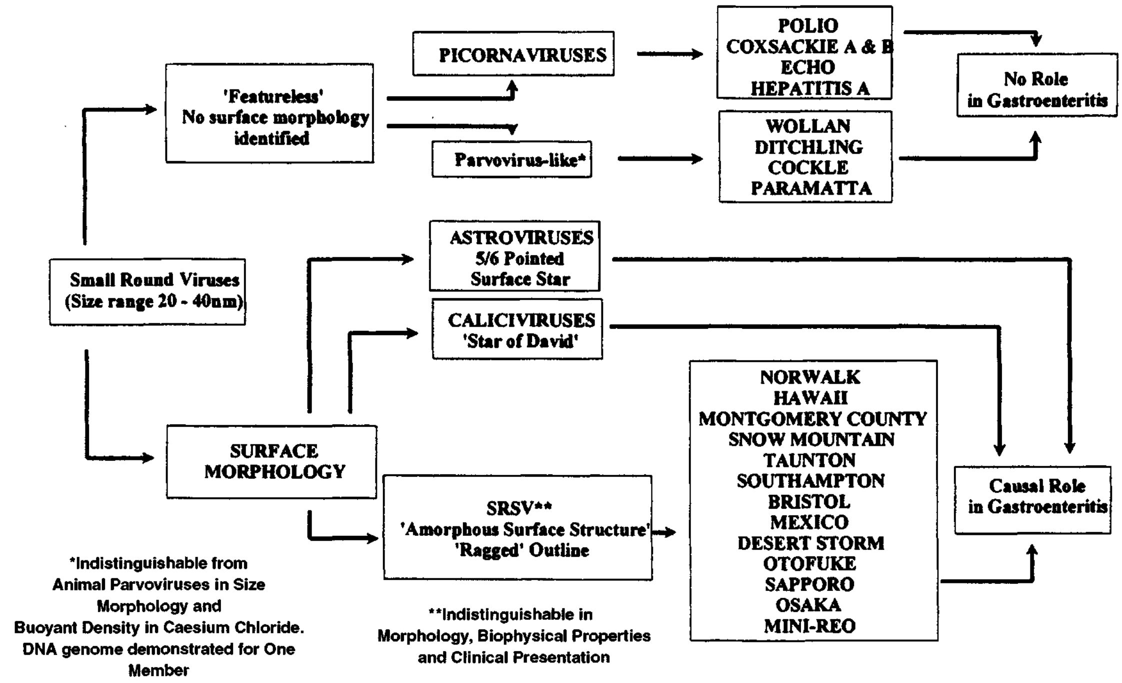
Small Round Viruses (Size range 20 - 40nm)
'Featureless' No surface morphology identified
PICORNAVIRUSES
Parvovirus-like*
POLIO COXSACKIE A & B ECHO HEPATITIS A
WOLLAN DITCHLING COCKLE PARAMATTA
No Role in Gastroenteritis
SURFACE MORPHOLOGY
ASTROVIRUSES 5/6 Pointed Surface Star
CALICIVIRUSES 'Star of David'
SRSV** 'Amorphous Surface Structure' 'Ragged' Outline
NORWALK HAWAII MONTGOMERY COUNTY SNOW MOUNTAIN TAUNTON SOUTHAMPTON BRISTOL MEXICO DESERT STORM OTOFUKE SAPPORO OSAKA MINI-REO
Causal Role in Gastroenteritis
*Indistinguishable from Animal Parvoviruses in Size Morphology and Buoyant Density in Caesium Chloride. DNA genome demonstrated for One Member
**Indistinguishable in Morphology, Biophysical Properties and Clinical Presentation

Figure 52–1 Electron Microscopical Classification of Human Small Round Fecal Viruses

tion, but cases are highly infectious. Occasional reports have, however, documented severe dehydration in some elderly patients, which required correction through the administration of intravenous fluids. Deaths arising from SRSV infections are unusual and have not been directly attributed to this group of viruses.[121]

Calicivirus. Symptomatic calicivirus infection occurs most commonly in infants and young children where it has been considered indistinguishable from the milder forms of rotavirus infection[55–57,61–65,213] Following transmission, there is an incubation period of 1 to 3 days, with symptoms persisting for an average of 4 days. Diarrhea and vomiting are common, whereas a fever with upper respiratory symptoms is less pronounced. There is no evidence that caliciviruses replicate in respiratory mucosal cells or that respiratory spread occurs. Because respiratory symptoms have been described mainly in young children, it is possible that they arise from concomitant respiratory viral infections, which are common in young children. Limited adult volunteer studies have been carried out where the virus was administered by the nasal/oral route.[55] Symptoms ranged from inapparent or mild to moderately severe and the latter included nausea, vomiting, diarrhea, pyrexia, and abdominal pain. The clinical presentations observed in these volunteer studies are compatible with those seen in naturally acquired infections.

Astrovirus. Astrovirus infection in man is followed by an incubation period of 3 to 4 days before the patient presents with symptoms of gastroenteritis. Affected patients present with systemic signs and symptoms including a fever (37° C), diarrhea, headache, nausea, malaise, and, uncommonly, vomiting.[135] Adult infections tend to be more severe than pediatric infections. These clinical features are similar to other viruses that cause gastroenteritis and are not clinically diagnostic.

52.3.2 Pathogenesis of Viral Gastroenteritis

SRSV. Virus replication is considered to occur within the mucosal epithelium of the small intestine despite the fact that direct evidence of such events has not been demonstrated. Ultrastructural studies, by light and electron microscopy, of jejunal biopsies from volunteers infected with Norwalk virus showed a partial flattening and broadening of the intestinal villi with disorganization of the mucosal epithelium.[2,206] The lamina propria was infiltrated with mononuclear cells and vacuolation of mucosal epithelium was noted. A crypt cell hyperplasia was a common finding, presumably in response to villous epithelium damage. Mucosal epithelial cells showed dilatation of the rough and smooth endoplasmic reticulum with an increase in multivesiculate bodies. Microvilli markedly were shortened and amorphous electron-dense material was present in the expanded intercellular spaces. Of considerable interest in these studies was the absence of recognizable virus particles in damaged mucosal cells. Similar results at the light microscopy and ultrastructural level also have been described from studies of volunteers infected with the Hawaii agent.[72,207] Overall, SRSV infections appear to cause a mild villous atrophy arising from limited virus replication in mucosal epithelium. These histopathological studies also demonstrated that the appearance of mucosal lesions in the small intestine was paralleled by a decrease in brush border enzymes that returned to normal levels in convalescence.[2]

Calicivirus. Unlike astroviruses, SRSVs, and other enteric viral pathogens, comprehensive ultrastructural studies of human caliciviruses in the gut have not been reported. Early studies in the UK demonstrated caliciviruses, by negative-staining electron microscopy, in the small intestinal gut lumen of a child who had died from gastroenteritis.[76] They were, however, unable to establish an etiological role because there were other complicating factors, and ultrastructural studies were not performed. An analogy with all the other diarrheal viruses would lead to the assumption that caliciviruses replicate in mucosal cells lining the villi of the small intestine. Extensive studies on the pathology of a bovine calicivirus-like agent (Newbury agent) have been carried out that demonstrated damage to enterocytes at the villus base.[94] The relevance of these studies to the pathogenesis of the classical human caliciviruses is unclear.

Astrovirus. Limited studies in man have shown that astroviruses infect the mucosal epithelium of the lower parts of the villi in the duodenum.[191] Crystalline arrays of astrovirus particles within the cytoplasm have been noted. The limited ultrastructural studies of astrovirus infections in man that have been reported are compatible with those observed in the small intestine of lambs that documented a transient villous atrophy.[209] In one study of human astrovirus infections, a short-term monosaccharide intolerance was reported[180] but this is not a serious sequel.

Virus replication within mucosal epithelial cells presumably results in cell lysis and release of astrovirus particles into the gut lumen where they become detectable in fecal samples. Virus excretion can be followed by diarrhea that is sometimes watery, although asymptomatic excretion is common. Diarrhea usually lasts for 2 to 3 days but may continue for a week or longer, and virus excretion persists while symptoms last. During the acute stage of the disease, 10^{10} particles per gram of feces can be detected in stool samples. Persistent symptomatic excretion has been documented for months in immunocompromised patients,[137] and such cases may be an important source for nosocomial infections.

Human volunteer studies have been carried out in an attempt to demonstrate that astroviruses are enteric pathogens. In these UK studies, 17 volunteers were infected with a fecal filtrate containing astroviruses and, subsequently, one volunteer developed diarrhea and a further four asymptomatic volunteers excreted astroviruses. These studies suggested that volunteers with pre-existing antibody prior to challenge were protected from symptomatic re-infection.[135,137] Although these adult volunteer studies are useful in assessing the potential of astroviruses as enteric pathogens and provide important clinical information, they are limited in assessing the overall burden of symptomatic primary infections in children, where the majority of infections occur.

52.3.3 Epidemiology of Viral Gastroenteritis (All Sources)

SRSV. SRSVs are now established as the most important cause of epidemic nonbacterial outbreaks of gastroenteritis throughout the world.[18,19,30,32,34,35,38,69,91,115,116,118,120,121,223] Extensive sero-epidemiological studies in the US, and the direct detection of SRSVs by electron microscopy particularly in the UK and Japan as well as elsewhere in the world, have reinforced the importance of these viruses in communitywide outbreaks, which often have significant economic importance. National initiatives directed toward the surveillance, ascertainment, and diagnosis by electron microscopy of UK outbreaks of nonbacterial gastroenteritis have recently shown that SRSVs are now a more common cause of outbreaks than salmonella and have provided important epidemiological data for many years. These data demonstrate an increasing prevalence of SRSVs in recent years with the suggestion of a winter seasonality (Figure 52–2) and show that all age groups are affected (Figure 52–3). This is epidemiologically different from that documented for caliciviruses and astroviruses, which demonstrates that these latter viruses are predominantly pediatric infections (see Figures 52–4 and 52–7).

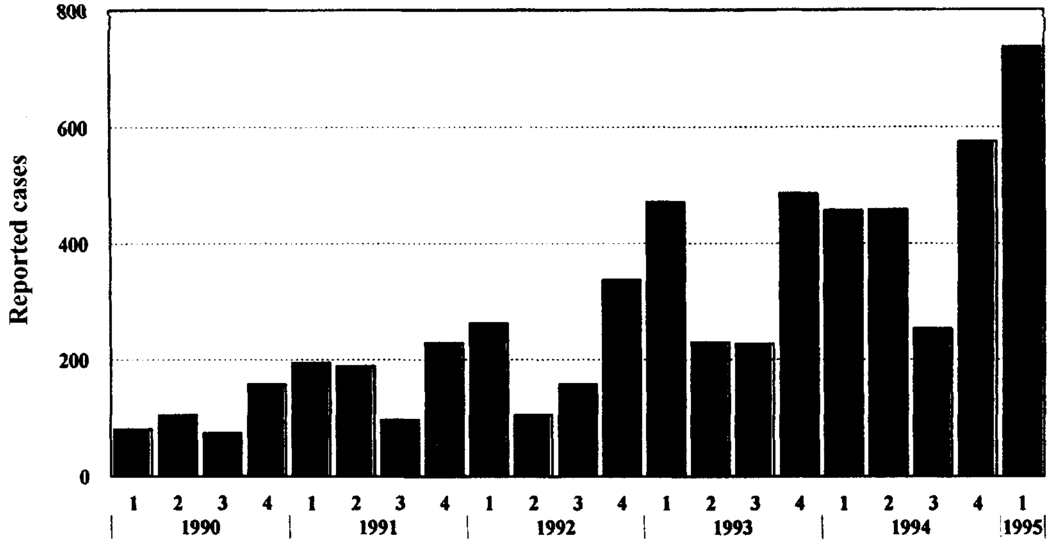

Figure 52–2 Total Numbers of SRSV in England and Wales by Quarter. Laboratory reports to Public Health Laboratory Service, Communicable Disease Surveillance Centre (CDSC) 1990–1995.

The diversity of settings associated with these economically important SRSVs is demonstrated by the early work in the US. The Norwalk virus caused a communitywide outbreak in Ohio, and the Hawaii and Montgomery County agents were responsible for small family outbreaks.[30,69,115,116,118] In contrast, the Snow Mountain agent caused an epidemic at a holiday camp in Colorado.[172] On the other side of the Atlantic, the Taunton agent was shown to cause considerable disruption in an orthopaedic and geriatric ward in southern England.[30,190] Subsequent surveillance and investigations of outbreaks in many parts of the world have identified the potential that SRSVs have for causing epidemic gastroenteritis in semiclosed or communitywide populations associated with families,[115,118] health care, holiday, and travel including cruise ships, schools, or higher education and the catering industry.[30,35,38,48,65,69,91,105,121,201,211] Epidemiological studies also have shown that outbreaks commonly occur in children and in adults, particularly the elderly, but less commonly in neonates or very young children.[18,30,35] These observations contrast with those of the classical caliciviruses that are rarely associated with epidemic gastroenteritis in adults. Although considerable data are available on the causal role of SRSVs in outbreaks, less is known about their endemic role in disease, which might be attributed to the short-lived and relative mildness of the presenting clinical features. As a result, hospitalization of individual cases is unusual and the opportunity to investigate sporadic cases is not usually possible.

Sero-Prevalence. The inability to propagate SRSVs in cell culture and the lack of a suitable animal model have severely limited sero-prevalence studies. American workers developed a number of serological assays, using fecal extracts containing Norwalk virus, to assess the acquisition of Norwalk antibody in populations in different countries.[91,115,117] Their studies showed that the presence of Norwalk antibody was acquired gradually during childhood with approximately 20% of children under 5 years old being positive. The prevalence of antibody in adolescence through early adulthood rose rapidly to 45% in 18- to 35-year-olds and 55% to 60% in 45- to 65-year-olds. This epidemiological picture is compatible with the natural history of SRSVs, which mainly cause outbreaks in older children and adults, and contrasts with that reported for astroviruses, caliciviruses, and rotaviruses. In contrast to the sero-prevalence documented in developed countries, acquisition of antibody to Norwalk virus in underdeveloped countries occurs at a very young age. Studies in Bangladesh and Ecuador showed that 75% to 100% of children had acquired antibody, while in Yugoslavia the sero-prevalence rate was intermediate between the US and less-developed countries.[115] Similar results have been re-

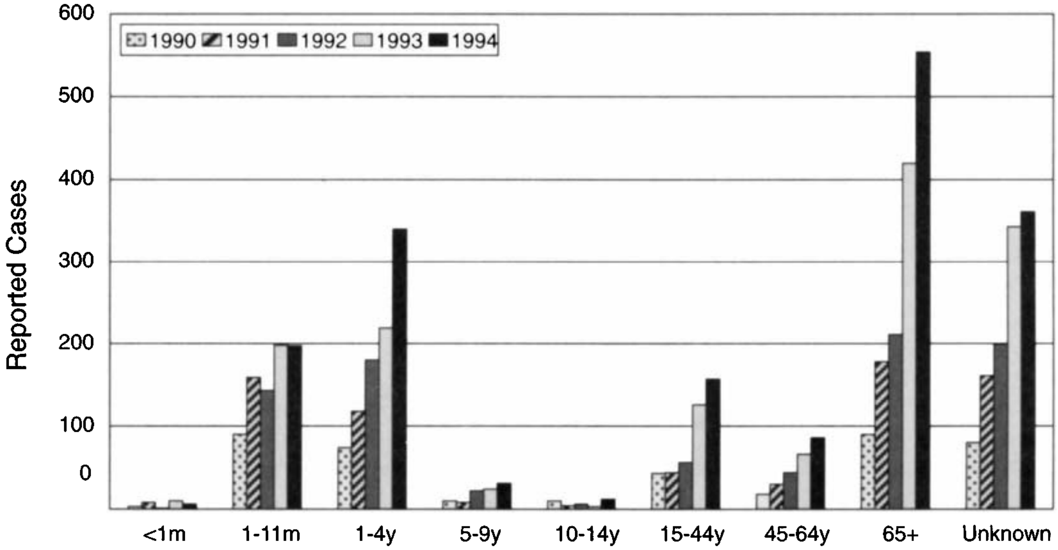

Figure 52–3 Total Numbers of SRSV by Age Group. Laboratory reports to CDSC for England and Wales 1990–1994.

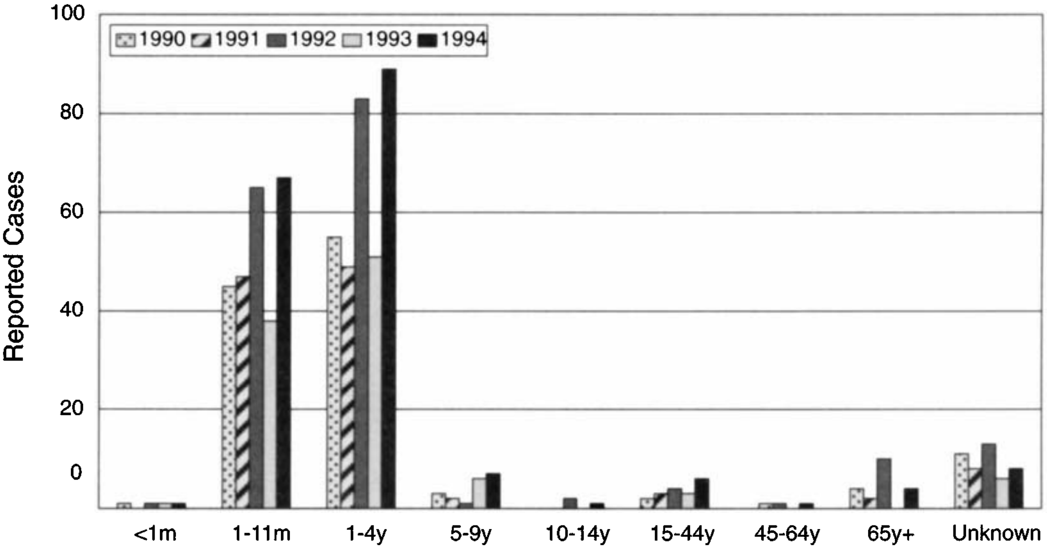

Figure 52–4 Calicivirus by Age Groups. Laboratory reports from all sources to CDSC for England and Wales 1990–1994.

ported from Taiwan and the Philippines. Reports of Norwalk virus antibody in blood donors from the US, Belgium, Yugoslavia, and Switzerland range from 54% to 77% and emphasize the ubiquitous nature of Norwalk virus in the world. Compelling evidence that Norwalk virus was responsible for many outbreaks throughout the world in the 1970s has been demonstrated by serological responses measured by a radioimmune assay (RIA).[115] All of these studies were carried out when Norwalk virus was circulating in the community. Current evidence, at the molecular level, suggests that Norwalk virus is no longer circulating in the US or in the UK and further sero-epidemiological studies using currently circulating SRSV strains as a source of antigen are now indicated.

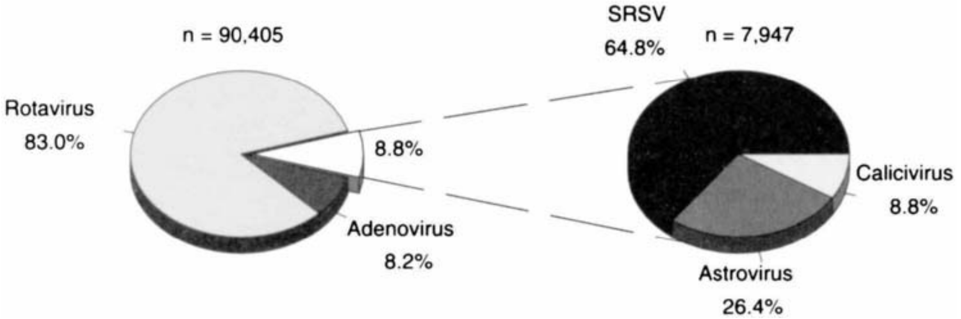

Figure 52–5 Enteric Viruses in England and Wales. Total number of laboratory reports from all sources to CDSC 1990–1994 denoting percentages attributable to rotaviruses, adenoviruses, and small round viruses.

The recent expression of Norwalk virus capsids in Baculovirus vectors is a major advance and has allowed further serological studies to be carried out on historical human serum using defined reagents in an enzyme-immunoassay (EIA) format.[218] Early results in the UK using these newly developed reagents suggest that approximately 25% of children aged 6 to 11 months old had Norwalk antibody and this increased to 90% in individuals over 60 years of age.[83] The high level of antibody to Norwalk virus documented in this report contrasts with the molecular epidemiology of SRSVs that has not identified Norwalk virus as a circulating strain in recent years. The possibility that these assays are detecting heterologous SRSV responses rather than specific Norwalk virus antibody should be considered.

Calicivirus. In common with the other diarrheal viruses, most of our data on the epidemiology of caliciviruses are derived from the detection of the virus by electron microscopy and limited sero-prevalence studies.

The first reports of a human enteric calicivirus came from studies carried out in Glasgow.[159-162] These workers were investigating the causes of gastroenteritis in children and detected classical calicivirus particles in both symptomatic and asymptomatic children. These studies failed to assign a causal role for caliciviruses in human disease because 20% of children excreting caliciviruses were asymptomatic. Subsequently, an outbreak of gastroenteritis in an infant and junior school in North London was investigated and caliciviruses were conclusively shown to be the causal agent.[61,63] This outbreak was described as winter vomiting disease, although spread to children in other classes in the school and to home contacts was negligible. The authors also postulated that spread to other children was limited because they were probably immune. These epidemiological and immunological features are not consistent with classical winter vomiting disease, which is now recognized as being caused by SRSVs. Since these early studies, evidence on a continual scale has incriminated caliciviruses in human enteric gastroenteritis and they are now established as enteric pathogens.[30,34,48,57,64,89,93,102,125] Numerous studies and surveys of sporadic cases of gastroenteritis in children have documented excretion rates from 0.9% to 6.6% in hospitalized children around the world.[35,57,61,125,159,163,169,213] A similar excretion rate (2.9%) has been reported in a US study in a daycare center.[165] All of these studies documenting calicivirus infection in hospitalized children suggest that, as astroviruses, they are a minor cause of clinically significant disease. Neonatal infection is often subclinical in contrast to that seen in older children and nosocomial spread has been described. The clinical importance of human enteric caliciviruses in the UK has been addressed and analyzed from national data reported by electron microscopy units. During the course of surveillance between 1985 and 1987, caliciviruses accounted for 0.9% of the total number of positive identifications from all sources of diarrheal viruses reported.[170] Over 90% of positive calicivirus identifications (1990–1994) were documented in children under 5 years of age (Figure 52–4).

Analysis of more recent national data (1990–1994) in England and Wales shows that caliciviruses accounted for 0.8% of all positive diarrhea virus reports from all sources, which is remarkably consistent with the previous studies. Further analysis of data, taking into account only small round viruses associated with diarrhea during this study period, showed that caliciviruses account for 8.8% of all small round viruses reported (Figure 52–5). The conclusions drawn from these national studies are that caliciviruses are a minor cause of clinically significant disease in the UK and suggest that infected children are uncommonly admitted to hospital.

Human calicivirus infections usually occur as sporadic cases in young children, but community and nosocomial outbreaks have also been recognized. Outbreaks have been documented in America,[165] Japan,[47,181] Australia,[93] and England,[57,61-64,109,138] and were documented not only in children,[30,35,47,48,57,62,89,93,109,165,181,213] but also among elderly patients in nursing homes[64,84,89] where the attack rate in some outbreaks ranged from 50% to 70%. Outbreaks in adults and elderly patients appear to be unusual and may represent infection in the face of a waning immunity. A distinct seasonality has not been described for calicivirus infections and it is apparent from the British studies that this virus is endemic in the community (Figure 52–6).

Sero-Prevalence. Studies in Japan have demonstrated that antibody to calicivirus is rapidly acquired between 6 months and 2 years of age with a sero-prevalence of 30%. This rises to 65% in 2- to 5-year-olds. In older children and adults, approximately 90% have acquired specific antibody.[178,203] Similar results have been documented in sero-prevalence studies in the US.[179] Confirmation of this high seroprevalence using pooled gamma-globulin and serum samples has been reported from all continents.[48,57] The high sero-prevalence in early childhood is in agreement with the documented excretion of human caliciviruses in young children. These data also suggest that asymptomatic or at least clinically insignificant disease in childhood is common in view of the paucity of calicivirus reports from hospitalized children with diarrhea. Overall the rapid acquisition of both calicivirus and astrovirus antibodies in childhood is similar to that seen in rotavirus infections and markedly different from that reported for SRSVs.

Astrovirus. Most of our knowledge on the epidemiology of astroviruses is based on the detection of morphologically typical particles by electron microscopy[159,160,162,163] and on limited sero-prevalence studies. The virus has been detected and reported from all five continents,[30,35,135] where a close association with childhood diarrhea has been recorded.[18,30,34,48,65,89,102,135]

Early studies in Glasgow in the 1970s showed that 80% of babies excreting astroviruses were symptomatic, while 12% had no diarrhea.[159,163] Nosocomial spread is well documented, and many outbreaks of astrovirus gastroenteritis in infants and young children have been described in nurseries and pediatric wards.[11,18,30,35,48,65,89,102,128,135,138] In one large pediatric outbreak in Japan, over 50% of children were symptomatic,[128] and, in another outbreak in a children's ward in the UK, spread to nursing staff occurred,[11,138] indicating that susceptibility is not restricted to neonates and children. In nurseries and pediatric wards, astroviruses can become endemic as a result of continually introducing new, susceptible children. Asymptomatic excretion (5% to 20%) has been reported in both neonates and young children; this represents a significant source for further spread of the virus in semiclosed communities.

Within our current knowledge, astroviruses appear to be a relatively minor cause of gastroenteritis in young children and are only occasionally incriminated in adult disease. Detailed longitudinal studies on astrovirus infections in the community have only rarely been reported. Analysis of surveillance data in England and Wales over a 3-year period between 1985 and 1987, based on electron microscopical observations, showed that astroviruses are endemic within the community with distinct peaks in the winter.[170] This seasonality appears to be constant in temperate climates. In the UK studies, a striking age-related prevalence was noted where the majority of reported symptomatic infections occurred in children under the age of 5 (Figure 52–7).

In a separate longitudinal study of infantile gastroenteritis in London, astrovirus infection was reported in 2.8% of children under age 6 (50/1,802), but none in children between the ages of 6 and 12 years.[61] National surveillance data in England and Wales confirm that astroviruses appear to be a minor cause of viral gastroenteritis in the UK and emphasize the seasonal distribution (Figure 52–8).

In contrast, sporadic cases or outbreaks in adults rarely are reported. In one outbreak in a convalescent home for the elderly in Marin County, California, 51% of residents developed gastroenteritis and the outbreak persisted for 3 months.[184] Although the virus was initially reported as a Norwalk-like agent, the published micrographs suggested an astrovirus morphology;[30] this has subsequently been confirmed.[101] An unusually large outbreak of gastroenteritis affecting both students and teachers has been reported by Japanese workers.[182] Sporadic cases in young adults also occur, which give rise to moderately severe diarrhea.

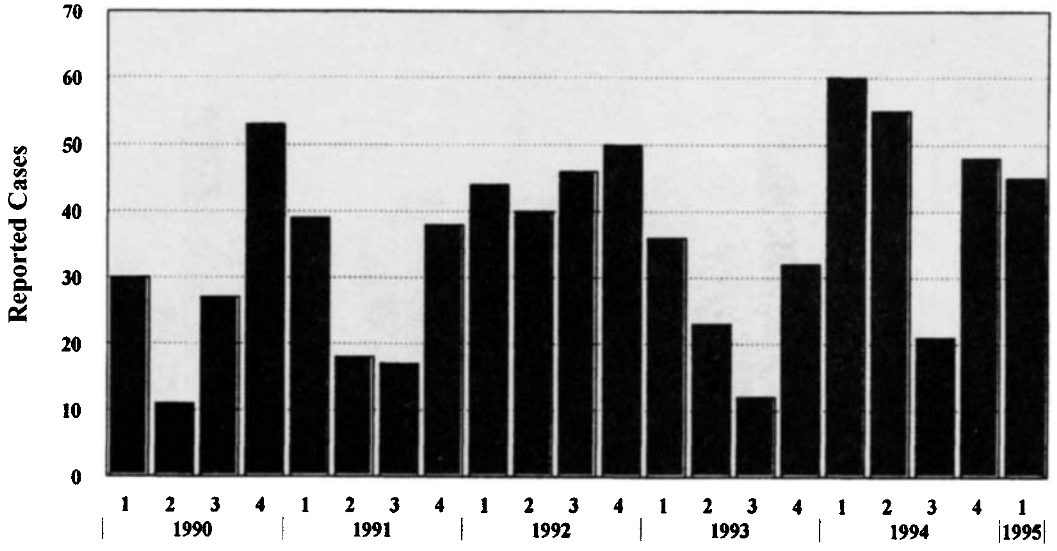

Figure 52–6 Caliciviruses in England and Wales. Laboratory reports to CDSC 1990–1995.

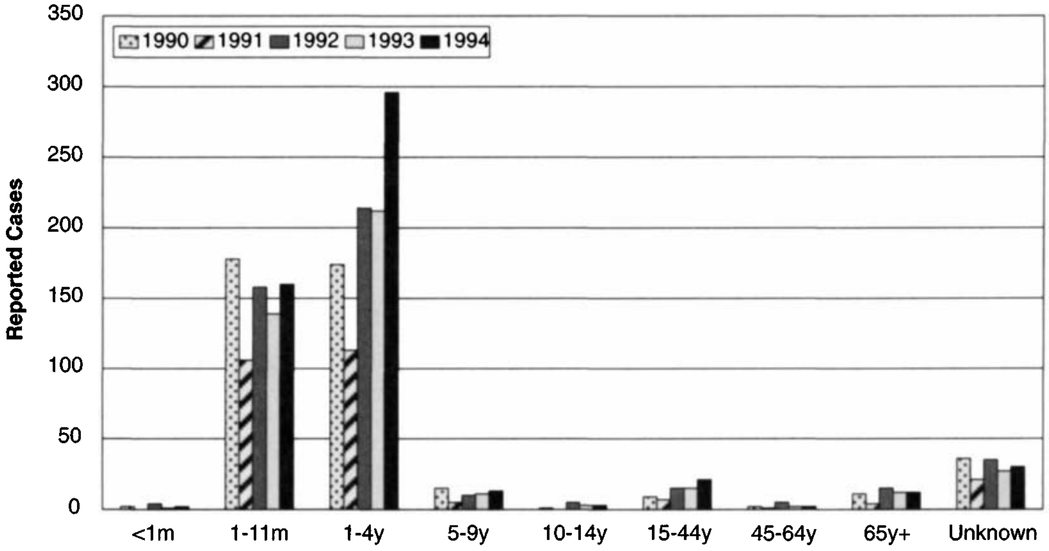

Figure 52–7 Astrovirus by Age Groups. Laboratory reports from all sources to CDSC from England and Wales 1990–1994.

Sero-Prevalence. Limited antibody prevalence studies have reinforced the observations that astroviruses are a minor cause of clinically significant disease in young children. One study in Oxfordshire, England, showed that 64% of 3– to 4-year-olds had been infected; this prevalence rose to 87% in 5- to 10-year-old children.[136] These data are compatible with the epidemiology of astrovirus infections, which demonstrate that the majority of infections occur in young children (Figure 52–7). This high prevalence of antibody in young children and the relatively low percentage of reported

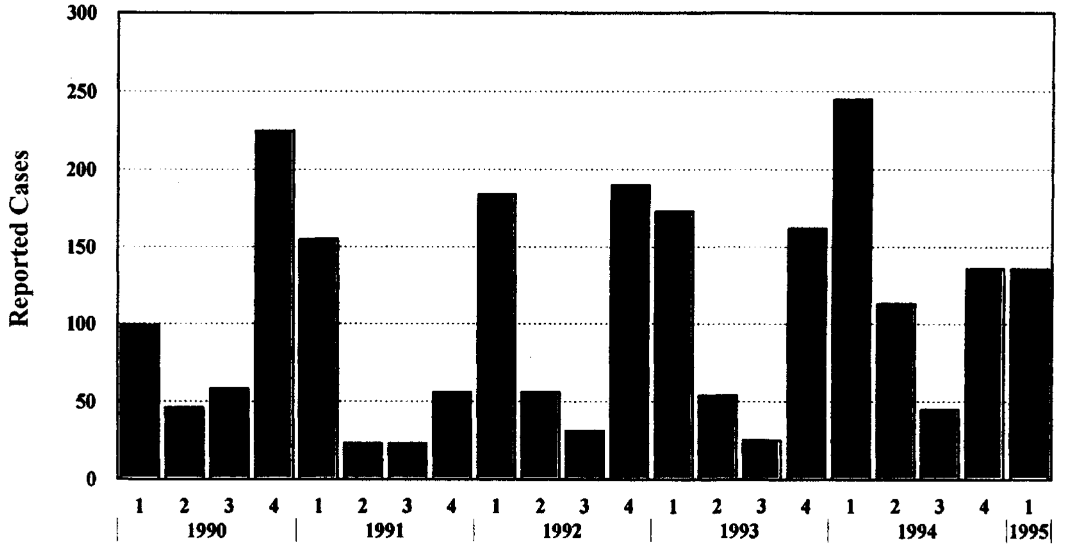

Figure 52–8 Seasonal Distribution of Astrovirus in England and Wales, Laboratory Reports from All Sources to CDSC 1990–1995.

astroviruses from symptomatic cases suggest that many infections are either subclinical or so mild that medical intervention is not required.

Some workers have suggested that the difficulties associated with the definitive identification of astroviruses in human fecal samples by electron microscopy has underestimated their role in viral gastroenteritis and that the recent application of molecular techniques has significantly increased their detection in clinical samples.[229] Other studies have suggested that astroviruses are second in importance to rotaviruses as a cause of viral gastroenteritis in young children.[89,102] These conclusions, however, are not sustainable unless comparable techniques are applied to all other enteric viruses, which in turn are likely to increase their detection.[35]

52.4 VIRAL HEPATITIS

Viral hepatitis in man is caused by at least six taxonomically distinct viruses classified as types A through F.[168,194] Two of these viral agents (A and E) are recognized as enteric infections and are transmitted either by the fecal/oral (Hepatitis A) or waterborne (Hepatitis A and E) route. These routes of transmission have obvious implications for foodborne human disease.

Hepatitis A originally was recognized more than 2,000 years ago when it was described as infectious icterus,[130,131] reflecting the appearance of jaundice in affected patients. Subsequently, the term viral hepatitis type A was proposed by McCallum in the 1940s[157,158] and this was adopted worldwide. The term infective or infectious hepatitis is commonly used in Europe and the US to denote the same syndrome. Extensive worldwide outbreaks of Hepatitis A have been recorded over the last three centuries involving many thousands of people, particularly during the course of World Wars I and II and civil wars.[130,131,168] Human volunteer studies were pivotal to the characterization of the disease and its distinction from Hepatitis B virus.[106] All the early attempts to propagate Hepatitis A in cell culture or in laboratory animal models, including a variety of nonhuman primates, failed and were reported at a symposium in 1954 in the US.[130]

In the 1970s, three important breakthroughs occurred in the study of Hepatitis A infection. Firstly, Hepatitis A virus was visualized for the first time as a result of using immune electron microscopy for the examination of stool samples from infected individuals.[75] These workers described the presence of 27 nm particles that were complexed by the addition of human antibody from patients convalescing from the disease. These studies represented a milestone in the study of Hepatitis A infection in man, and the specificity of this virus as the etiological agent was confirmed by the demonstration of rising antibody titers in affected patients.[75]

The second important breakthrough came with the development of animal models[106] and the description of 27 nm particles in the liver of infected marmosets. Thus by the end of 1974, the agent of viral hepatitis type A had been visualized and an animal model had been described. These studies were fundamental to the expansion of our knowledge on the characterization of Hepatitis A virus[23,193] and its disease and led to the development of a variety of immunological assays used to study the epidemiology of this infection.[26] Finally the difficulties and frustration that many workers had experienced in attempting the in vitro propagation of Hepatitis A in cell cultures were overcome.[192] These studies, demonstrating the growth and passage of Hepatitis A virus in cell culture, were fundamental to its characterization and were the springboard for the development of a safe inactivated human vaccine that is now widely available.

Epidemics of human hepatitis unrelated to either Hepatitis A or B (non A, non B, non C hepatitis) recently have been recognized in Asia, Africa, and Mexico.[15,196] Although the disease initially resembled Hepatitis A, it became clear that this was not the etiological agent as a result of serological tests that excluded both Hepatitis A and B. Thus a new human disease culminating in a hepatitic clinical presentation was recognized that was transmitted by the fecal/oral route.[9,15,16,20] Studies in Soviet Central Asia by IEM identified for the first time a virus etiology for this new form of human hepatitis[16] by aggregating virus particles in human fecal samples with specific antibody; subsequently, the agent has been designated Hepatitis E virus (HEV).[21] Animal models have been important in the study of Hepatitis E infections in man;[24] these have included the use of cynomologous monkeys, owl monkeys, macaques, and tamarins.[25,129] These studies have established that HEV is the etiological agent of non-A non-B hepatitis in man by the demonstration of seroconversions in human cases and in experimentally infected primates.[8,20–22,24,27]

Outbreaks of Hepatitis E in man have only recently been recognized,[15,20–22,24,29,40,234] largely because of the difficulties associated with its specific laboratory identification and the inability to propagate the virus serially in cell culture. Progress is now being made arising from the molecular characterization of the viral genome,[199,214,215] which suggests that it is a member of the Caliciviridiae family. Hepatitis E is currently recognized as an important health problem in Asia, Africa, and Central America[15,21,29,40,222,234] in contrast to its low prevalence in the US and Western Europe. Recent reports have identified Hepatitis E infections among American travelers to Mexico, India, Pakistan, and Nepal.[40]

Although Hepatitis E is currently not a recognized public health problem in the UK, US, or Western Europe,[205] waterborne outbreaks have been described in developing countries.[21,40,216,234] To date, foodborne outbreaks have not been documented. Hepatitis E may therefore become an important food- or waterborne disease in developed countries in the future. Routine laboratory methods are not yet available for Hepatitis E, but future methods will certainly include molecular epidemiological studies and laboratory diagnosis of affected patients by the demonstration of specific IgM responses. Recent studies have demonstrated the application of Hepatitis E baculovirus-expressed capsid proteins in the development of an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-HEV antibodies.[132,153,216,220] This assay will provide a means of establishing the sero-epidemiology of Hepatitis E virus in developed as well as developing countries. Now that the viral genome has been characterized, molecular methods can be developed (polymerase chain reaction [PCR]) to investigate putative water or foodborne outbreaks in the future.

52.4.1 Clinical Aspects of Hepatitis

Hepatitis A. Infection with Hepatitis A virus is followed by an incubation period of 10 to 50 days, which is dose-dependent. The outcome is extremely variable, ranging from asymptomatic or subclinical infection (common in children) to anicteric hepatitis (without jaundice) or icteric hepatitis (jaundice). Symptoms can range from mild to severe and prolonged. Prior to the onset of hepatitis, the patient commonly develops anorexia, fever, malaise, fatigue, myalgia, nausea, and vomiting. Affected patients are infectious prior to the onset of hepatitis and for a few days after the development of jaundice. The excretion of Hepatitis A virus in infected individuals prior to the onset of clinically recognizable disease is an important factor in foodborne transmission.

Pathogenesis. Natural infection with Hepatitis A virus follows ingestion of the virus by the fecal/oral route. The acid stability of the virus allows passage through the stomach and it enters the host via the gastrointestinal tract. From this point of entry, the virus replicates within hepatocytes in its primary site—namely the liver. At the peak of virus replication, there is little liver damage, suggesting that other mechanisms are involved in the pathology of Hepatitis A infection. Although not yet conclusive, it has been suggested that liver damage may result from immune mechanisms after viral replication.[106,195]

Epidemiology. Worldwide, Hepatitis A is one of the most common causes of infectious jaundice, and recurrent epidemics are well recognized.[106,168] Rates of infection are highest in children and young adults in low socioeconomic groups in developing countries. Sanitation, polluted water supplies, and poor personal hygiene are major factors in the spread of the virus; in these settings, where overcrowding also occurs, infection in the young can reach 100%. Person-to-person spread by the fecal/oral route is the major mode of Hepatitis A transmission. There have been more than 35 outbreaks in man where infection occurred from close contact with infected primates (usually chimpanzees). Human cases in these outbreaks have usually involved the animal caretaker.[106]

In contrast, in populations in the developed world that have adequate sanitation, less crowding and facilities for improved hygiene, infection occurs in older age groups. This essentially means that in modern developed societies, large segments of the population remain susceptible. In these situations, large outbreaks can and do occur following the introduction of the virus into a community. As subclinical infection is common in young children, it is worth emphasizing that the first indication of a Hepatitis A outbreak in a community may be symptomatic infection in adults following exposure to their asymptomatic young children. Spread of the virus in young schoolchildren in modern societies often occurs because of a reduction in standards of personal hygiene in the school environment. In the UK, many of the Hepatitis A infections that occur in older children and adults result in several clinically recognized diseases.[103] Three main patterns of disease can be recognized, namely sporadic cases, often in adult travelers returning from countries with a high endemicity,[210] common-source outbreaks (food related), or large community outbreaks that evolve slowly. In the latter case, school-age children and young adults predominate where spread has occurred by the person-to-person route. Between 1987 and 1991, the annual notification rate for Hepatitis A in the UK increased from 3.6 to 14.6 per 100,000 population; however, although the disease occurs throughout the world, incidence figures are unreliable because most cases are either mild or subclinical and are therefore not reported.[168]

Sero-Prevalence. Hepatitis A antibody acquisition is directly related to the standards of sanitation, personal hygiene, and overcrowding. In underdeveloped countries, exposure occurs at a very young age and sero-prevalence may reach more than 90% in the first decade of life. Thus considerable geographical variation in incidence occurs with wide differences in sero-prevalence between different countries.[168] In modern societies with improved sanitation, infection is less frequent, and more than 50% of the population may reach adult life without any evidence of exposure as determined by antibody prevalence studies. Clearly this will be dependent on the socioeconomic groups investigated. Sero-prevalence studies in the UK (1986–1995) have shown that a higher proportion of young children were immune to Hepatitis A in the north of England than in the south,[103] suggesting greater exposure in the north of the country. These sero-epidemiological studies in the UK and also in the US have shown that the incidence of Hepatitis A infection has fallen in the last decade and that there is the considerable potential for future epidemics as the majority of the adult population remain susceptible.[103,168] Food- or waterborne transmission of Hepatitis A to a largely susceptible population in the developed world is an obvious future threat, giving rise to the potential for large communitywide outbreaks.

52.5 BIOPHYSICAL PROPERTIES AND VIRUS INACTIVATION

In general SRSVs, astroviruses, caliciviruses, and Hepatitis A virus are resistant to lipid solvents, detergents, extremes of pH, and moderate temperatures. All are sensitive to free chlorine or hypochlorite solution; their properties are shown in Table 52–2. Viruses that infect and multiply in mucosal cells of the gastrointestinal tract are acid-stable. This is an important property that allows these viruses to pass through the stomach before reaching the small intestine. As a result of their acid stability, they will survive food processing and preservation conditions designed to establish the low pH that inhibits bacterial and fungal food-spoilage organisms. SRSVs have not been propagated in cell culture; much of our knowledge on their biological properties is derived from human volunteer studies. In one such study, an SRSV (Norwalk virus) retained its infectivity after exposure to pH 2.7 for 3 hours at room temperature. It was also shown to be stable after treatment with 20% ether at 40° C for 18 hours.[71] Like most viruses, SRSVs, astroviruses, caliciviruses, and Hepatitis A retain infectivity after refrigeration and freezing and outbreaks of SRSVs and Hepatitis A relating to frozen foods are well documented. It is noteworthy that those food storage conditions selected for their inhibition of bacterial and fungal organisms are ideal for the maintenance of virus infectivity. All of the above viruses are inactivated by normal cooking processes, but SRSVs in particular are not always inactivated in shellfish after minimal heat treatment. Volunteer studies demonstrated that one strain of SRSV (Norwalk virus) was not inactivated when heated at 60° C for 30 minutes. Additionally, it is unclear whether this virus would be completely inactivated after many pasteurization processes. Studies in the UK have led to recommendations that the internal temperature of shellfish meat should be raised to 90° C and maintained for 1.5 minutes to inactivate Hepatitis A virus.

SRSV survive well on inanimate surfaces, as demonstrated by the persistent outbreaks that have been documented in hospitals, residential homes, and particularly on cruise ships. Although chlorine-based disinfectants are the choice for decontamination of environmental surfaces and are effective for all enteric viruses, SRSVs are resistant to 3.75 to 6.25 mg chlorine l^{-1} which is equivalent to free residual chlorine of 0.5–1.0 mg l^{-1} present in drinking water. SRSVs are inactivated by 10 mg chlorine l^{-1}, and this is more than is required to inactivate polio virus or human rotavirus.

52.6 MODES OF TRANSMISSION

52.6.1 Person to Person

SRSV. Human transmission of SRSVs occurs through the fecal/oral route and has been convincingly demonstrated by the American volunteer studies.[115] An infectious dose as low as 10 to 100 virus particles is considered sufficient to initiate infection. Despite these observations and the lack of evidence for secondary spread by the respiratory route, it is clear that many of the explosive outbreaks described cannot be solely due to fecal/oral transmission. Electron microscopy studies have shown that SRSVs can be detected in vomitus[30,34] and the projectile vomiting associated with acute infection is an additional explanation for the transmission of SRSVs, either from aerosolized virus or through the contaminated environment, and has been emphasized in a number of recent reports.[31,33,34,44,105,204] The high energy associated with projectile vomiting induces aerosolization of virus particles and would disseminate infectious virus into the immediate environment. This, in conjunction with the low infectious dose, allows the spread of infection to secondary contacts and would explain the acute, explosive outbreaks that are commonly documented in semiclosed communities. Environment contamination through the dissemination of infectious vomit is particularly important to the transmission of

Table 52–2 Biophysical Properties of Foodborne Viruses

			Inactivation by:				
Virus	*Nucleic Acid*	*Size*	*pH≤3*	*Ether-Chloroform*	*Alcohol 70%*	*60° C**	*Free chlorine ≥ 10 mg l^{-1}*
SRSV[†] Norwalk/ Norwalk-like	RNA	32 to 38 nm	No	No	Partially	No (30 min)	Yes
Calicivirus	RNA	32 to 38 nm	No	No	Partially	No (30 min)	Yes
Astrovirus	RNA	28 ± 1 nm	No	No	Yes	Yes (10 min)	Yes
Hepatitis A	RNA	27 to 32 nm	No	No	Yes	No (60 min)	Yes

* Dependent on suspending media

[†] Most data derived from studies on Norwalk virus

SRSVs in food premises. The natural history of SRSV infections should now include not only person-to-person spread by the fecal/oral route, but also mechanical transmission (hand to mouth) arising from projectile vomiters who have contaminated the immediate environment. The likelihood of spread arising from the inhalation of aerosolized virus by contacts in close proximity to the index case has been suggested but not proven definitively.[31]

Calicivirus, Astrovirus, and Hepatitis A. Person-to-person spread through the fecal/oral route is considered the major mode of spread for all these viruses. Vomiting as a vehicle for transmission has not been demonstrated. Hepatitis A is also excreted in urine and this can represent an additional dimension for spread to close contacts.

52.6.2 Foodborne Transmission

Outbreaks of nonbacterial gastroenteritis have occurred in a wide range of defined settings, such as holiday camps, cruise ships, health care services, schools, and nursing homes where the identification of the mode of transmission is not always possible. Many of these outbreaks result from person-to-person spread through the fecal/oral route or by mechanical transmission (hand/mouth) from the contaminated environment. Projectile vomiters have an important role in the dissemination of infectious virus (SRSVs) by aerosolization that in turn contaminates the immediate environment. Air currents within a confined area will compound the spread of the aerosols generated by symptomatic patients.[31,33] All these factors explain the explosive outbreaks that have been recorded in the literature.

In addition to the mode of spread of SRSVs described above, there are clearly other transmission routes that give rise to point source outbreaks. These will include transmission of SRSVs through the food chain, and current evidence suggests that this mode of spread, although not quantifiable, is significant and of considerable public health importance. Although foodborne SRSV viral gastroenteritis is the most significant viral problem to the food industry, it is also clear from the literature that Hepatitis A has been commonly reported while other viruses, such as the astroviruses and caliciviruses, are only rarely incriminated in foodborne incidents.

Shellfish. The cultivation of bivalve mollusks is a major industry worldwide. Many of the estuaries and inlets used to cultivate shellfish also are used for sewage disposal. Filter-feeding bivalve mollusks concentrate sewage and retain human microbes present in the sewage. Depuration processes are known to be ineffective in removing human viruses from contaminated shellfish and, as a result, significant public health problems arise from human consumption of the raw or undercooked product.[45,124,221]

Many outbreaks of viral gastroenteritis implicating raw shellfish have been described from Europe,[6,37,46,78,98,144] the US,[41,43,74,127,173] Japan,[122] and Australia.[175,176] One of the largest outbreaks associated with raw shellfish occurred in Australia where more than 3,000 people were affected.[175,176] Recent studies in Japan[122] identified 715 foodborne outbreaks out of a total of 908 outbreaks investigated; raw oysters accounted for 45% of these foodborne outbreaks. SRSVs were identified as the commonest virus, accounting for 92% of all viral foodborne outbreaks in this study. In many outbreaks, it was recognized that the shellfish had been harvested from sewage-contaminated waters. The total reported burden of gastroenteritis associated with the consumption of shellfish in England and Wales between 1981 and 1990 is shown in Figure 52–9. It is apparent from these data and from laboratory investigations that viral gastroenteritis is the predominant risk factor associated with eating shellfish. Outbreaks of shellfish-associated viral gastroenteritis in the UK and worldwide in which a pathogen has been identified have been shown to be predominantly caused by SRSVs, which identifies this group of viruses as a major cause of foodborne viral gastroenteritis.

It is also apparent that in many shellfish-related outbreaks a pathogen is not identified. It is worth considering the reasons for these observations as most patients in these outbreaks present with symptoms, which are consistent with viral gastroenteritis if the Kaplan criteria are used to assess the etiology of an outbreak.[120,121] It is well recognized that many outbreaks of shellfish-associated gastroenteritis are either reported too late, or not at all, for appropriately timed clinical samples to be submitted to allow definitive laboratory investigations to be carried out. Furthermore, not all laboratories have the expertise or electron microscopes to achieve a diagnosis in affected patients, and often samples are submitted too late to allow optimal detection of SRSVs. It follows from this that most, if not all, of these outbreaks of gastroenteritis of unknown etiology are likely to be due to SRSVs, which further emphasizes the importance of this group of viruses in shellfish-associated human disease. The recent development

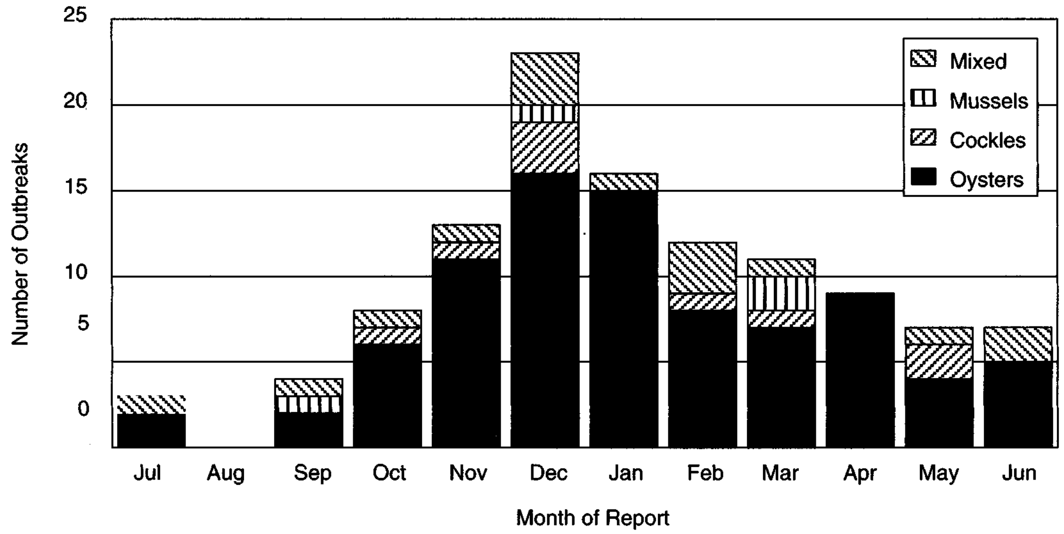

Figure 52–9 Outbreaks of Viral Gastroenteritis due to Molluskan Shellfish in England and Wales 1981–1990

of more sensitive molecular methods for the detection of SRSVs[34,50,113,139] will resolve this problem in the future. Thus, the available data represent considerable under-reporting, at least in the UK, but probably throughout the world.

The epidemiological evidence that uncooked or partially cooked shellfish represent a significant risk to man is now overwhelming. In spite of this, it is apparent that the number of human cases of viral gastroenteritis arising from eating shellfish is an underestimation of the actual problem. Until recently, this was partly due to the difficulties associated with the laboratory diagnosis of SRSVs by electron microscopy, which is relatively insensitive. With the recent molecular characterization of SRSVs has come the real possibility of improving detection methods for this difficult group of viruses. Historically it has not been possible to detect SRSVs in contaminated shellfish because of the insensitivity of electron microscopy. The application of the PCR to extracts of shellfish has now shown that contamination with SRSVs and other enteric viruses can be demonstrated.[12–14,79,143,144,146,147,212,219,233] Furthermore these molecular techniques can be used as powerful epidemiological tools to monitor the spread of SRSVs in contaminated shellfish to the general population and vice versa.[79,212] Recently, elegant molecular epidemiological studies in the US have shown that shellfish grown in clean waters were contaminated by a fisherman who had an acute, explosive episode of diarrhea in close proximity to the oyster bed. Sequencing data from the RNA polymerase region of SRSVs isolated from the fisherman and from patients who subsequently ate the contaminated oysters showed that the isolates were identical.[79] These are the first studies to be reported on the use of molecular epidemiology to monitor the spread of SRSVs in contaminated shellfish through the community and demonstrate one application for this technology. Future studies using molecular techniques will increase the detection of SRSVs in human fecal samples from outbreaks of shellfish-associated nonbacterial gastroenteritis and will reduce the number of outbreaks where an etiological agent has not been identified.

Shellfish contamination arising from polluted water also has been well documented with Hepatitis A virus. Numerous outbreaks have been recorded in the UK, the US, and other parts of the world.[39,53,67,95,96,164,168,200] In the years 1980–1981, seven shellfish-associated outbreaks of Hepatitis A occurred in the UK. The largest outbreak of Hepatitis A arising from the consumption of contaminated shellfish was recorded in Shanghai in 1988.[95] This resulted in over 30,000 reported cases of Hepatitis A in young adults who ate uncooked clams grown in polluted water. Hepatitis A transmission from shellfish is now rare in the UK as a result of heat treatment of cockles prior to being sold to the consumer.

In addition to shellfish, two outbreaks of Hepatitis A resulting from contaminated raspberries have also been documented in the UK over a 12-year surveillance period.[6] Recently, an outbreak of Hepatitis A related to contaminated strawberries[169] occurred in Michigan where 153 students and teachers became ill. It was considered that as many as 9,000 youngsters and adults in Los Angeles may also have been exposed. The contaminated strawberries originated from Mexico and may have been contaminated at source. Additionally, an unusual outbreak of Hepatitis A in the UK relating to bakery products (bread) that affected 50 people also has been reported.[226]

Shellfish-associated outbreaks of astroviruses and caliciviruses also have been documented but only rarely. In one oyster-associated outbreak involving 39 naval officers, one-third developed diarrhea within 4 days of eating the oysters. Astroviruses were detected in stool samples from 5 affected patients and IgM antibodies were present in two patients.[30]

Presumably, reports on foodborne astrovirus and calicivirus infections are rare because of the high level of immunity in the general population. Thus it is clear that any of these viruses can be transmitted to man and resultant disease or resistance is dependent on previous exposure and immunity from childhood infection. SRSVs are the single exception where immunity is short-lived,[34,115,186] and reinfections commonly occur. This explains the significant role of SRSVs in foodborne viral gastroenteritis.

Food Handlers. Outbreaks of viral gastroenteritis caused by SRSVs have also commonly involved the contamination of raw or cold food by an infected food handler; this appears to be a widespread occurrence worldwide.[6,32,37,52,53,79,92,97,121,122,133,134,154,156,190,197,223,227] A wide range of foods has been implicated in foodborne outbreaks that have been extensively reviewed[32,52–54,79,92,97,115,121,122,134,156,223] and include salads, bakery products, sandwiches, hamburgers, fresh fruit, cold meat, and other cold foods. The source of this contamination has often identified a symptomatic food handler in these outbreaks.[38,190,197] The available evidence strongly suggests that those food items that require the most handling present the greatest risk. Surveillance of foodborne outbreaks in Minnesota identified 51 outbreaks over a 10-year period between 1981 and 1991. Contaminated cold food items were identified as a source in 45 (88%) of these outbreaks.[97] Overall a food handler source was identified in 74% of foodborne outbreaks of viral gastroenteritis in Minnesota. The importance of point source outbreaks is that the secondary attack rate is often high (>50%), which in turn amplifies the original foodborne outbreak.

The high infectivity of SRSVs and their potential for infecting large numbers of people through the food chain also has been demonstrated by an outbreak in a bakery[133] where a food handler, who was symptomatic while working, contaminated a large batch of butter cream frosting, which was used to make 10,000 frosted food items. Human consumption of these food items gave rise to 3,000 cases of viral gastroenteritis. In a recent occurrence in the UK, an infected food handler prepared a custard mix that was used to make custard slices. More than 100 people who consumed these custard slices developed viral gastroenteritis (SRSV), and it was concluded that as many as 2,000 people may have been affected (unpublished).

Outbreaks arising from food handlers who become ill after preparing or serving an implicated meal also have been reported.[154] Such outbreaks have also been documented where food handlers were apparently asymptomatic or convalescent from an episode of gastroenteritis.[227] As a result, it is not always clear in every outbreak how food handlers transmit SRSVs through the food chain. Pertinent to these questions is the natural history of SRSV infections in man. The spectrum of illness in affected patients can range from a mild, self-limiting infection to a more severe clinical presentation. Vomiting is a significant symptom and can occur in more than 50% of affected patients. As a result, there is considerable potential for symptomatic food handlers to contaminate (vomit) the immediate working environment or adjacent area (ie, rest rooms or toilets). This environmental contamination would then represent a source of contamination and infection to other food handlers. There is a strong possibility that, in these circumstances, mechanical transmission (hand/mouth to food) might occur, and given the high infectivity of SRSVs, this could explain those outbreaks involving food handlers who were not ill. Whatever the exact mechanism of transmitting SRSVs is, in truly asymptomatic food handlers, these occurrences are a minority and most of the outbreaks described have identified a food handler who was ill at the time of preparing the food or had been ill shortly before. It is also acknowledged that symptoms can be so mild that food handlers may not recall any illness but they would, nevertheless, be highly infectious. Outbreaks relating to food-handlers who are convalescing from gastroenteritis are rare, but this emphasizes the need for food handlers to remain at home for at least 48 hours after recovery from the original illness.

Prospective studies in Bristol, UK, over a 6-year period have shown that approximately 23% of all

SRSV laboratory-confirmed outbreaks are food related.[32] Statewide surveillance data from Minnesota suggested that of the 34 foodborne outbreaks that occurred over a 3-year period (1981–1983), 12 (35%) were caused by SRSV.[97] Recent national surveillance data in the UK for a 2-year period (1992–1993) documented 1,275 outbreaks of gastroenteritis where 50% were considered to be foodborne (639 outbreaks) and show that SRSVs accounted for the majority of the viral (47 outbreaks) foodborne outbreaks (Figure 52–10). This study identified that a food handler was suspected of contaminating the food in 14 of the 41 outbreaks caused by SRSVs.[156] Thus the variation in foodborne outbreaks documented by different workers in the world may reflect the particular interests of those workers. The difficulties of ascertainment, investigation, and laboratory diagnosis all contribute to the under-reporting of foodborne viral gastroenteritis, which is recognized by most workers in the field. Thus, in addition to outbreaks arising from person-to-person spread and mechanical transmission from the contaminated environment, SRSVs are also commonly associated with point source outbreaks. It is now clear that in many cases a likely food vehicle for transmission, other than shellfish, has been identified.

Food handlers also have been identified in the transmission of Hepatitis A to food that was not subsequently cooked further. In one incident a food handler was identified as the source of a foodborne outbreak on a floating restaurant in Florida,[155] and in a separate incident in the UK, bread was incriminated that was thought to have been contaminated by a food handler.[226] Extensive foodborne outbreaks also have been documented in a hospital in Vermont[217] and at a Naval training center in San Diego.[107] In one outbreak in Jefferson County, Kentucky, 202 cases of Hepatitis A occurred after exposure to contaminated green salads,[202] and in a further outbreak 54 people became ill after eating in a restaurant in Tennessee. The source of this latter outbreak appeared to be a food handler who was an intravenous drug abuser.[39] All of these outbreaks of Hepatitis A involve a diversity of foods and emphasize that contamination can occur through any food that is handled either without cooking (raw) or after cooking by an infected food handler.

Surveillance of Hepatitis A outbreaks in the UK has identified seven outbreaks involving a food handler over a 12-year period. The main problem in food handlers is that infected individuals are excreting high levels of virus prior to the onset of classical signs and symptoms of hepatitis infection, and transmission may therefore occur before intervention measures can be instigated. The transmission of astroviruses and caliciviruses by an infected food handler has not been definitively demonstrated to date.

Waterborne Transmission. Transmission of SRSVs, caliciviruses, astroviruses, and Hepatitis A infections to man through the waterborne route have not been definitively demonstrated in the UK. In contrast, contaminated water supplies in America such as municipal water, semi-public water, drinking water on cruise ships, recreational swimming water (lake or pool), and commercially produced ice cubes, all have been incriminated in SRSV outbreaks.[18,28,65,91,97,115,121,142,172,230] A number of point source outbreaks of Hepatitis A also have been described by American workers as a result of fecally contaminated drinking water.[106,174] There is considerable interest in establishing the importance of waterborne infections in the UK and Europe and the application of molecular technology will provide definitive data in the future.

52.7 LABORATORY DIAGNOSIS

52.7.1 Electron Microscopy

Historically, electron microscopy has been the major tool used for the examination of clinical samples from patients with viral gastroenteritis and has had a major role in elucidating the etiology of this syndrome. It is not used for the examination of patients with viral hepatitis (Hepatitis A) because maximum fecal excretion occurs before the onset of jaundice and the virus has no distinguishing features in the electron microscope to allow separation from other small, round, featureless viruses (picornaviruses and parvoviruses) present in fecal samples. The diagnosis of Hepatitis A infections in man has been traditionally made by serological methods (see Section 52.9). It must be emphasized that electron microscopy cannot be used for the detection of viruses in food as it is too insensitive.

SRSV. Currently, electron microscopy is the most commonly used laboratory diagnostic method for the direct detection of SRSV in stool samples. All SRSVs are indistinguishable in the electron microscope when examined by standardized negative staining methods (1.5% phosphotungstic acid pH 6.5). Virus particles possess an amorphous surface structure, lacking a defined symmetry, with a ragged outline (Figure 52–11B). This ragged outline prob-

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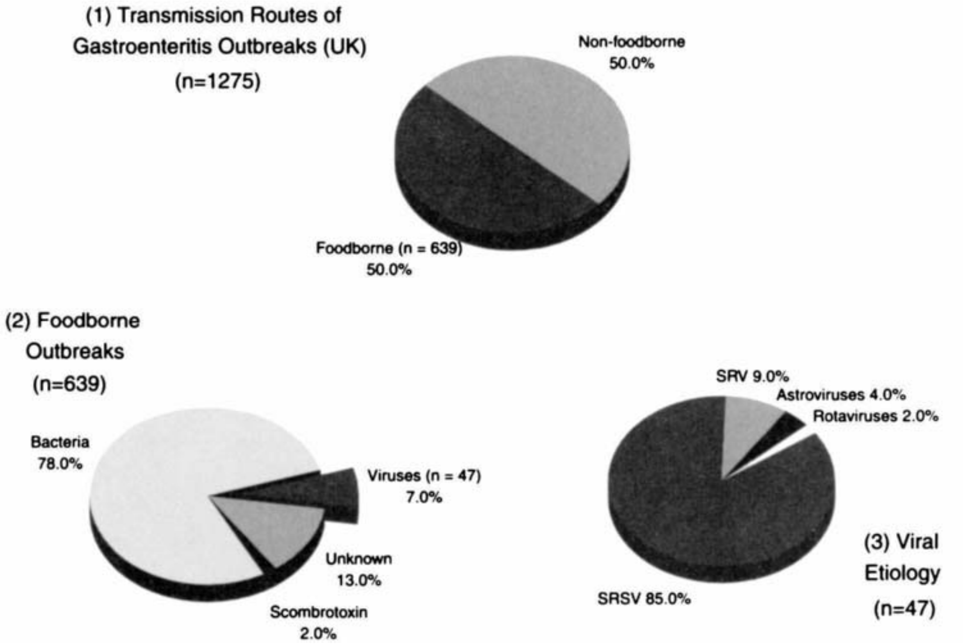

Figure 52-10 Total Number of Outbreaks of Gastroenteritis in the UK (1992–1993). Data show that half of the total numbers of outbreaks are foodborne with viruses accounting for 7%.

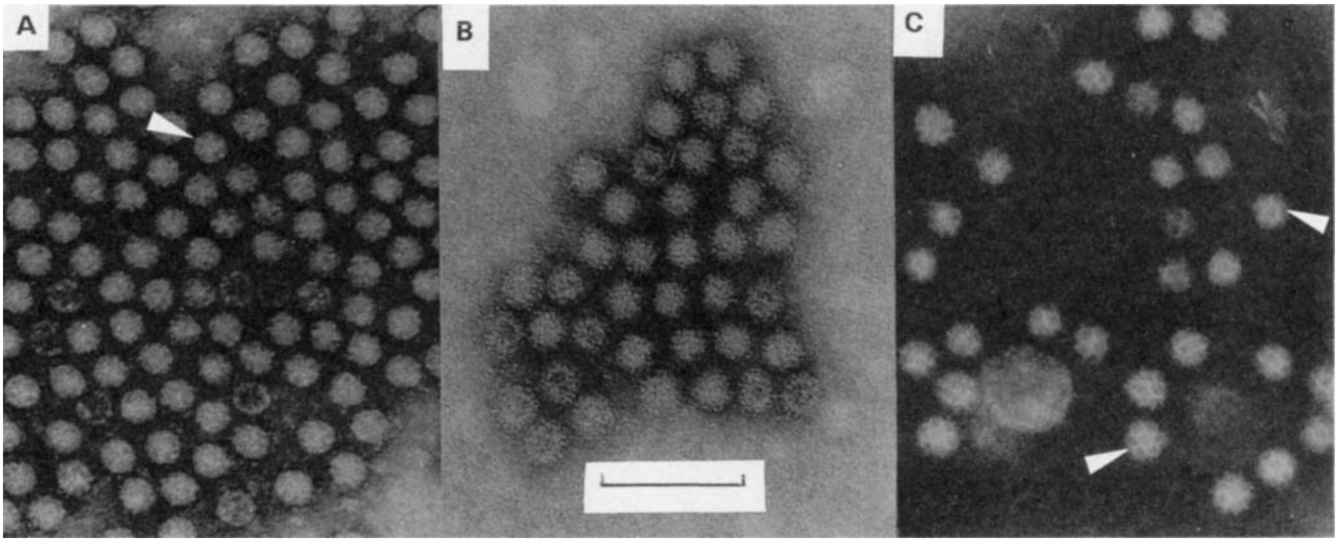

Figure 52–11 Electron Micrograph of Small Round Fecal Viruses. **A**, Astrovirus (arrow indicates surface "star" morphology); **B**, SRSV (Norwalk-Like); **C**, Classical calicivirus (arrows indicate surface cups). All stained with 1.5% phosphotungstic acid pH 6.5. Bar = 100 nm.

ably explains the wide range of particle diameters (32 to 38 nm) described. It is essential that preparations of SRSV are examined in the absence of antibody, because its addition can lead to masking of the surface structure of virus particles and inappropriate identification.[30,36] SRSVs commonly are excreted in stool samples as small aggregates and at low levels, often below the number that can be detected by the electron microscope (10^6 viruses g^{-1} of stool) and this necessitates detailed and careful examination of electron microscopic preparations. Contamination with nonviral proteins in clinical samples often obscures SRSVs and this can be overcome by applying the method of solid phase immune electron microscopy (SPIEM) using human convalescent sera.[37] Samples prepared in this way are far superior to conventional methods of virus concentration used in electron microscopy and increase the detection rate of SRSVs significantly.

Recent comparative studies have shown that overall SPIEM is almost as sensitive as molecular methods for detecting SRSVs[8] with the added advantage of speed that is so important to the management of foodborne outbreaks. Electron microscopy also has the added value of being a catch-all system where all viruses associated with gastroenteritis can be detected. It is likely that electron microscopy will retain an important role in the investigation of human stool samples from foodborne SRSV outbreaks where rapid diagnosis is a prerequisite.

Antigenic Variation. Cross-challenge studies in volunteers have demonstrated that Norwalk and Hawaii viruses are antigenically distinct.[115,218,231] Further human volunteer studies using the Montgomery County agent and Norwalk virus identified a one-way cross protection. These early studies also demonstrated that a short-term homologous immunity occurred after challenge in humans with all three viruses.[70,115,231] Long-term immunity to homologous challenge has only been demonstrated in some patients,[186] and this unusual aspect and complexity of the immunobiology of SRSVs is currently under investigation.[115]

The antigenic relatedness of Norwalk, Hawaii, and Snow Mountain viruses has been studied by assessing the pattern of sero-responses in volunteers.[115] Using an EIA assay variable degrees of antigenic relatedness were observed, with a two-way cross-reaction between Snow Mountain and Hawaii viruses and a one-way cross-reaction between Norwalk and Snow Mountain viruses documented.[115] These results may reflect heterologous responses and contrast with the greater specificity of IEM or SPIEM, where relatedness is based on intact virions and capsid epitopes.

Using immune electron microscopy (IEM or SPIEM), four SRSV antigenic types have been identified in the UK[139,149,150] and six in the US,[151] while Japanese workers have identified nine serotypes.[183] Recent suggestions are that the nine Japanese serotypes may include similar viruses on the basis of cross-reactions and may therefore be an overestimate of the actual number of distinct serotypes. Definitive data on the antigenic relationship of SRSVs will have to await the development of specific monoclonal antibodies rather than human convalescent or hyperimmune sera that are more broadly reactive. This is a reality now that some strains of SRSVs have been completely sequenced and will allow the production of baculovirus expressed viral proteins and the development of monoclonal antibodies. In spite of these difficulties, SPIEM has been extremely rewarding in defining the changing prevalence of different SRSV antigenic types in the UK and for identifying appropriate isolates for molecular characterization.

Calicivirus. Human enteric caliciviruses originally were described in fecal samples using negative staining techniques.[161] This family of viruses possesses a unique morphology. Classical particles viewed along their threefold axes of symmetry possess a central, stain-filled cup (calices) surrounded by six peripheral cups giving rise to a Star of David appearance (Figure 52–11C). Calicivirus particles viewed along their twofold axes of symmetry possess four cups and also are identifiable definitively. Particles viewed along their fivefold axes present as a 10-pointed sphere and may not be so readily identified as the classical particles described above. Unlike astroviruses, which possess a smooth entire edge, caliciviruses possess a ragged outline that makes accurate measurements difficult. This explains the particle diameters of 31 to 38 nm quoted by most workers.

This unique morphology allows definitive calicivirus identification by electron microscopy,[160] but electron micrographs always should be taken to record observations made from viewing specimens in the microscope and allow more detailed inspection. This unique calicivirus morphology is the only acceptable criterion for identifying the classical human caliciviruses and any divergence from this morphology is not acceptable. Occasionally, difficult specimens arise and when the morphology is suggestive of a calicivirus, but not convincing, then replicate samples should be prepared and re-examined. Electron microscopy is currently the only routine method available for laboratory diagnosis and requires skilled operators to achieve consistently reproducible results.

Antigenic Variation. Human enteric caliciviruses possessing classical morphology have been grouped into four distinct antigenic types by IEM and share a common antigen detectable by RIA.[57,59]

Astrovirus. Astroviruses originally were recognized by Madeley and Cosgrove in 1975[162] and subsequently described in detail a few years later.[160] These workers coined the term astroviruses as a result of the characteristic six-pointed star morphology (Figure 52–11A), which is resolved on the surface of virus particles by electron microscopy following negative staining. The negative stain (1.5% phosphotungstic acid pH 6.5) used in electron microscopy is important for the consistent resolution of the surface star. Astroviruses are remarkably uniform in size measuring 28nm (± 1nm) in diameter and possess a smooth, entire, sharply delineated outer edge. The characteristic star-shaped surface morphology, which is enhanced in high-contrast micrographs, is present on 10% to 20% of particles and is diagnostic, allowing definitive morphological differentiation from calicivirus with which they have been confused. Often astroviruses are present in large numbers in electron microscopical preparations made from samples collected during the acute phase of the illness. Naturally occurring, large or small aggregates are common and probably arise from the release of crystalline arrays from infected cells that have lysed. The possibility that these virus aggregates result from antigen/antibody complexes (copra antibody) has not been confirmed and is an unlikely explanation. Procedures used in the concentration of astroviruses from clinical samples prior to examination by electron microscopy are important. Astroviruses are unstable if they are pelleted from cesium chloride gradients but purification and stability in tartrate/glycerol gradients prior to examination by electron microscopy have been demonstrated.[10]

Difficulties arise in the identification of astroviruses if IEM is applied. Antibody-mediated aggregation of astrovirus particles in suspension masks the typical star-shaped surface structure and leads to inaccurate identification as demonstrated by the original classification of the Marin County agent as a Norwalk-like virus.[184] In contrast, the capture of astrovirus particles onto antibody-coated electron microscope grids (SPIEM) is an elegant and highly sensitive method of identification and has allowed the diversity of astrovirus capsids to be subtyped into seven serotypes.[135] The major disadvantage of immune electron microscopy is its high specificity in comparison with direct electron microscopy, which remains the only catch-all technique available for all diarrhea viruses.

Antigenic Variation. Studies in Oxford, England, using SPIEM and immunofluorescence identified six antigenically distinct astroviruses over a 10-year period.[135,145] Serotyping of 165 strains documented serotype 1 as the most prevalent strain (64%). The authors also demonstrated that the frequency of the other serotypes varied from year to year. Since this report, one additional serotype has been recognized. Herrman and colleagues recently have developed a monoclonal antibody enzyme-immunoassay and have confirmed the antigenic variation described by the Oxford Group.[100]

52.7.2 Enzyme-Immunoassay (EIA)

The need to develop alternative assays for the detection of human small round viruses in stool samples has been recognized by many workers. Japanese workers developed a solid-phase RIA using a hyperimmune guinea pig serum and demonstrated that this assay was more sensitive than electron microscopy for the detection of caliciviruses.[177] Subsequently, these workers converted this assay to an EIA that had a similar sensitivity.[179] These assays are not yet commercially available, but will presumably be available in the future for investigating clinical samples.

Recent studies have described the identification of an astrovirus group antigen that is shared by five of the known astrovirus serotypes. The preparation of monoclonal antibodies to the group antigen allowed the development of an EIA, which detected all astroviruses grown in cell culture.[100] More recently, these workers have modified their EIA to detect astroviruses in stool samples[101] and have compared this assay with IEM. Their studies have shown that the EIA has comparable sensitivity and specificity to IEM and it is likely that this assay will eventually replace electron microscopy in diagnostic laboratories. The assay will allow more extensive studies to be carried out in the future on clinical samples and will enhance our knowledge on the epidemiology and medical importance of astroviruses.

More recently, the molecular characterization of Norwalk virus and other SRSVs has allowed the expression of capsid proteins in a baculovirus expression system.[50,68,111-113,139,148] The use of these reagents has resulted in the preparation of specific antibodies for use in an antigen capture EIA.[81,112] Preliminary results suggest that these assays are serotype specific and represent a major advance in the development of alternative assays to electron microscopy for the detection of SRSVs. Nevertheless, it is unlikely that any of the above EIA developments will be sufficiently sensitive for use in the direct detection of viruses in food incriminated in foodborne outbreaks of viral gastroenteritis or viral hepatitis.

52.7.3 Molecular Diagnosis

SRSV and Calicivirus. With the complete sequencing and characterization of the SRSV and calicivirus genome[68,113,139] has come the prospect of applying molecular techniques to clinical diagnosis and transmission patterns[88,123] and possibly to the detection of viruses in food. Although methods now have been described where the PCR has been applied to the RNA polymerase region of SRSVs, continual refinement and design of new primers have been necessary because of the diversity of this group of viruses. Studies to date, surprisingly, have demonstrated that the RNA polymerase region of SRSVs shows considerable heterogeneity and that SRSVs can be classified into two genotypes.[3,50,85-87,225] Thus a continual assessment of SRSVs at the molecular level may be necessary at least in the short term. Molecular epidemiology and the sequencing of SRSV strains will be a powerful tool in future investigations of point source outbreaks particularly for the examination of shellfish[3,13,14,35,50,144,147,169,212] and possibly other implicated foods and in monitoring spread of SRSVs between hospitals.[88] Because foodborne outbreaks caused by classical caliciviruses are rare, the development and application of primers for this virus are less urgent than for SRSVs. These techniques currently are restricted to the research laboratory but future developments will ensure an important role in routine diagnosis from clinical samples where they will complement electron microscopy. An important future requirement is the removal of PCR inhibitors from stool samples to ensure maximum sensitivity.

Astrovirus. The complete sequence of one strain of astrovirus has been published,[228] but the application and potential for molecular diagnosis has not yet reached diagnostic laboratories. A sensitive dot-blot hybridization procedure for detecting astroviruses in fecal samples has also been described[229] but has not been validated in comprehensive field studies.

Molecular technology, although difficult, is likely to be the only method by which any of these viruses, as well as Hepatitis A, can be detected directly in food or water samples incriminated in outbreaks. The method has the additional benefit of allowing precise fingerprinting of the virus by sequence analysis, thus providing definitive molecular evidence of the role of any of these viruses in human foodborne outbreaks.

52.8 VIRUS ISOLATION

52.8.1 SRSV

At the time of writing, there are no reports that document the isolation of SRSVs in either cell or human intestinal organ cultures. Developments in this area clearly are needed to study the cell tropism, cellular receptocytes, and viral replication of this important group of viruses. However, it is unlikely that isolation in cell cultures will ever be used as a method for diagnosing infected individuals or for the examination of food samples as in vitro methods are too slow and labor intensive.

Numerous studies in a wide range of animals have failed to identify a suitable model.[115] Some success has been achieved with the transmission of Norwalk virus in chimpanzees where serological responses and the excretion of Norwalk antigen in stool samples was described,[115] but animals have no role in diagnosis of foodborne infections.

52.8.2 Calicivirus

In contrast to the serial propagation of astroviruses in trypsin-containing media, similar attempts to isolate calicivirus have failed. A single report documents that human enteric caliciviruses produced a nonproductive infection in dolphin kidney and in human embryonic kidney (HEK) cells in the presence of trypsin.[57,58] Cytoplasmic replication was demonstrated by immunofluorescence and by radio-labeling experiments. This report has not been confirmed, and many other workers have failed to propagate human caliciviruses in vitro.

52.8.3 Astrovirus

Initial studies demonstrated that astroviruses undergo an incomplete replication cycle in HEK cells, but serial passage was unsuccessful. The addition of trypsin in serum-free medium has allowed the replication of a productive infection with release of infectious progeny virus that can be serially passaged.[135] In the absence of trypsin, astrovirus replication results in the production of a 90,000 molecular weight protein in the cell cytoplasm.[50] In the presence of trypsin, this protein is cleaved into three smaller proteins that appear to be essential to the production of infectious progeny virus.[50,229] Serial passage has been reported in trypsin-treated HEK and a continuous line of Rhesus monkey kidney cells (LLCMK2), and more recently in a cell line established from a human colonic carcinoma (CaC0-2).[229] Virus yields in CaC0-2 cells are approximately 2 logs greater than in the former two cell lines. The ability to propagate astroviruses in cell culture allows sero-epidemiological studies to be carried out, but this is not the method of choice for diagnostic purposes or for the examination of food as it is labor intensive, expensive, and likely to be less sensitive than immunologically based assays.

52.9 DIAGNOSTIC SEROLOGY

52.9.1 SRSV

American workers have been successful in using reagents derived from human volunteers to carry out both sero-epidemiology and diagnostic serology in outbreaks of Norwalk virus.[115] The development of an RIA, EIA, and immune adherence assay (IAHA) has greatly facilitated study of the natural history of Norwalk virus infections and has provided compelling evidence on its etiological role in epidemic gastroenteritis in the 1970s.[115] With the recent molecular characterization of SRSVs, reagents derived from humans and experimentally infected animals will have a limited future utility.

The expression of Norwalk virus capsids in baculovirus vectors has been used in the development of an EIA for the identification of Norwalk virus antigen and antibody in naturally occurring or experimentally induced infections.[83,115,218] However, the demonstration of high prevalences of Norwalk virus antibody in recent serological studies using these baculovirus expressed viral proteins[83] has produced results that are difficult to interpret because Norwalk virus is no longer circulating in the community. The possibility that this assay is detecting heterologous responses should be considered. In general, the development and application of reagents arising from the molecular characterization of recent isolates of SRSVs will considerably advance our knowledge of the epidemiology of foodborne infections as they will represent currently circulating strains of SRSV. However, it is unlikely that methods directed toward a serological diagnosis will be useful in diagnostic laboratories in the future to investigate foodborne infections where rapid diagnosis is required for establishing the etiology of the outbreak and for the instigation of control measures. They will, however, be useful for retrospective diagnosis and establishing the etiology of an outbreak.

52.9.2 Calicivirus and Astrovirus

Serology has been applied to the diagnosis and confirmation of both calicivirus and astrovirus infections. Studies have shown that IgM responses or rising titres (IgG) occur following calicivirus infection[47,57,62–64,179] and astrovirus infections in children.[11,135] These techniques, although useful in epidemiological studies, are not ethically justified for the diagnosis of enteric diarrhea virus infections in young children, particularly as other noninvasive and sensitive methods such as electron microscopy and PCR are available for rapid diagnosis.

52.9.3 Hepatitis A

Diagnostic serology is the standard method used universally for the diagnosis of Hepatitis A infections in man. The demonstration of Hepatitis A specific IgM in early acute/convalescent serum samples is a reliable and sensitive method and has provided an important diagnostic assay for establishing the importance of Hepatitis A within the viral hepatitis syndrome in man. The demonstration of abnormal liver function tests in serum samples complements the specific IgM assay. Assays for the detection of IgM also have been developed for urine and salivary samples; these have facilitated the investigation of outbreaks.[110,187] Sero-prevalence studies directed toward the detection of specific IgG also have been used widely in many countries as the mainstay of Hepatitis A epidemiology.[17,77,103,104,108,126,185,208]

52.10 PREVENTION AND CONTROL

SRSV viral gastroenteritis and hepatitis A are the most important viral causes of foodborne disease in man. Other viruses such as astroviruses, caliciviruses, and, to a much lesser extent, rotaviruses, are extremely rare as a cause of foodborne illness. It is apparent that all these enteric viruses do not replicate in food or water and their presence in the food chain is directly related to human fecal contamination.[52] This is true of shellfish grown in polluted waters, drinking water contaminated by human sewage, or the mechanical transmission of these viruses into food directly by infected food handlers. It follows that prevention and, therefore, control of these foodborne infections[6,37,38,66] can be interrupted by the food and shellfish industry given an appropriate level of education and a change in current practices. This will include changes necessary to reduce contamination of the environment through sewage disposal.

52.10.1 Shellfish Transmission

The bivalve mollusks (shellfish) are clearly a major source of outbreaks of foodborne transmission of viral gastroenteritis in developed countries and, to a lesser extent, Hepatitis A. Attempts to grow shellfish in clean waters (sewage free) have been occasionally instigated and this action will remove all enteric viruses and will significantly reduce this public health hazard from the food chain.[79] Unfortunately even when shellfish are grown in clean waters, contamination has occurred through discard of human sewage by local fishermen collecting the shellfish.[79] Thus the education of fishermen collecting shellfish is important to maintain clean oyster beds. It is clear that many shellfish beds are contaminated by sewage and remain a public health hazard; therefore, the shellfish should always be purchased from accredited suppliers to reduce the risk of viral transmission. Current methods of depuration are ineffective in eliminating viral contamination, and cooking shellfish renders them unpalatable to the consumer and has not gained universal favor. Steaming shellfish also has been studied and shown to be ineffective in inactivating SRSVs.[124] Nevertheless, heating shellfish (cockles) in the UK has eliminated Hepatitis A outbreaks. Cross contamination of other foods in the kitchen from contaminated shellfish also is well recognized, and shellfish need to be discarded when food-related outbreaks occur.

52.10.2 Waterborne Transmission

Waterborne transmission as a result of human fecal contamination will presumably continue to occur occasionally in the developed world as a result of processing errors in public drinking water supplies. However, such episodes are fortunately rare within our current knowledge. Shock chlorine treatment (>10 mg l^{-1} for 30 minutes) has been shown to be effective in eliminating infectivity from swimming baths associated with outbreaks of SRSV viral gastroenteritis,[38] and also is effective for the inactivation of Hepatitis A and other enteric viruses. Recent unpublished reports of high levels of astroviruses in bathing seawater in the UK may represent a public health problem but confirmation is urgently needed.

52.10.3 Food Handlers

The other important source of foodborne viral gastroenteritis and occasionally Hepatitis A transmission is the infected food handler. Symptomatic food handlers often have been incriminated in outbreaks of viral gastroenteritis, and they must be excluded from the work environment when symptomatic and for a minimum of 2 days after resolution of symptoms. It should be recognized that susceptible food handlers can prolong the risk in food establishments if other employees are symptomatic, because of the high risk of person-to-person spread. Decontamination of all environmental surfaces within the food preparation area is essential if food handlers have been ill on the premises.[37,38] This should include staff rest areas and toilet facilities. This is necessary to eliminate aerosolized vomit, generated by symptomatic food handlers, which has settled on hard surfaces, as a source of transmission from the contaminated environment. All high-risk foods that have required handling must be discarded following an outbreak among food handlers in a food establishment. The high infectivity of SRSVs, and the explosive outbreaks of gastroenteritis that commonly occur in closed or semiclosed communities, present a major challenge to control of infection and require immediate and aggressive intervention measures.[31,33,44] Transmission and spread of SRSVs occur via the fecal/oral route and by transport of aerosolized virus by air currents generated by projectile vomiting, which results in environmental contamination.[31,37] Measures directed toward the interruption of these modes of transmission must include enteric precautions and exclusion of symptomatic staff, but these alone are ineffective and also should include measures to deal with incidents in which employees vomit on the premises.

Effective hand washing by food handlers is a prerequisite in all settings relating to foodborne viral outbreaks and should be carried out routinely. Staff employed to clean and decontaminate food premises must wear disposable plastic gloves and gowns for cleaning vomit, fecal material (toilets), or contaminated clothing. Masks should be considered in all instances where aerosols are generated. Soiled linen or clothing should be handled carefully to reduce to a minimum the generation of aerosols, and transported in plastic bags for decontamination. All potentially contaminated environmental surfaces including toilets and rest rooms should be disinfected with a chlorine-based product and cleaned with a hot detergent solution.

Volunteer studies on the immunobiology of SRSVs have demonstrated that both short- and long-term protection occurs. Until the mechanisms involved in immunity have been demonstrated and fully understood, it is unlikely that a vaccine can be effective.[115] Effective vaccines have been developed for Hepatitis A virus, and its widespread introduction to those employed in the food industry would essentially eliminate foodborne Hepatitis A transmission by food handlers. This control measure is not currently considered to be cost-effective in the UK, although it has been recommended in America.

A significant and cost-effective measure for the prevention of foodborne viral infections relates to the current level of education among those employed in the food industry. The mechanisms involved in transmission should underpin prevention strategies and should be explained to all food handlers and emphasis placed on the single most important measure of personal hygiene, which includes regular hand washing, particularly after toilet use. Viral gastroenteritis among food handlers needs special attention by those responsible for the management of food safety. The absence of food employees with gastrointestinal sickness, however mild, should be positively encouraged; it should not result in a financial penalty and would be an additional effective measure appropriate to prevention.

52.11 CONCLUDING REMARKS

Analysis of foodborne virus infections from many countries has established that SRSVs are a major cause of epidemic gastroenteritis and their importance relates to the diversity of strains circulating and the lack of long-term immunity, which allows reinfections to occur at regular intervals. Other enteric viruses, such as the astroviruses and classical caliciviruses, predominantly are pediatric infections giving rise to long-term immunity that persists into adulthood and explains the rarity of foodborne incidences arising from these enteric infections. Similar arguments are relevant to rotavirus infections.

Common-source foodborne SRSV infections arise from two distinct situations. It is clear that infected food handlers are a primary cause of foodborne outbreaks and measures to intervene in the transmission chain are particularly relevant to control. The exclusion of food handlers who are symptomatic, however mild, for 48 hours after recovery is paramount as is the decontamination of food premises when outbreaks arise. Education of employees and the application of best practice within the food industry remains an important objective and needs to be promoted. Regular hand washing by food handlers is essential and an important procedure to reduce the transmission of the highly infectious SRSVs.

An equally important mode of transmission of SRSVs is through contaminated shellfish, particularly bivalve mollusks. Ideally, shellfish should be grown in pollution-free water, but this is not always feasible. The accreditation of shellfish beds will go some way toward resolving the hazard to humans but falls short of definitive control. Depuration procedures are known to be ineffective and heat inactivation prior to consumption, although successful for the control of Hepatitis A, renders the product unacceptable to the human palate. Screening of shellfish prior to human consumption using the recently described molecular methods does not inform the producers that infectious virus is present as these methods do not establish the presence of live or inactivated virus. Further research in this area to establish whether a relationship exists between genome detection and infectious virus is clearly required.

Although foodborne Hepatitis A infections are relatively uncommon in the UK, they are well recognized in other parts of the world. Transmission from food handlers can be interrupted by exclusion from the work premises when the employee is symptomatic. Some workers in the US have suggested that food handlers should be immunized, but cost-benefit analysis in the UK does not support this conclusion. Nevertheless it is clear that with the improvements in personal hygiene and the socioeconomic status of the population, sero-prevalence in the UK is declining in some regions and it will be important that surveillance procedures for foodborne Hepatitis A infections are robust in the future. Transmission of Hepatitis A through the human consumption of shellfish remains a problem in many parts of the world and can only be eradicated by propagation of shellfish in pollution-free water or by heat inactivation of the virus.

The increasing use of sewage sludge as a fertilizer and soil conditioner on agricultural land poses new problems in the future for the prevention of foodborne viral infections. Although there are no currently recorded outbreaks of foodborne viral infections associated with the use of sewage sludge on agricultural land, a recent report where rotaviruses and Hepatitis A were detected by ELISA and molecular methods in market lettuce in Costa Rica serves to identify a potential problem.[99] The increasing importation of produce into the UK and elsewhere in the world requires assessment in terms of the risk to the consumer. As the level of potential contamination currently is unknown, this is a clearly identifiable area for risk assessment. In the meantime, thorough washing of products grown on agricultural land where sewage sludge has been used is imperative to the delivery of a safe product. Research in this area is urgently required to determine the stability of human viruses in sewage and the risk to the human food chain. This is an emerging area applicable to the control of human disease and requires robust surveillance procedures throughout the world. Surveillance of foodborne viral infections worldwide generally is poor, and procedures should be taken to improve the detection and reporting of these infections. Only then can a comprehensive picture of human foodborne viral disease become established and better defined.

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53 Toxigenic Fungi and Mycotoxins

Maurice O. Moss

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53.1 INTRODUCTION

53.1.1 Fungi and Food

Fungi are a very diverse group of organisms and have a significant impact on the production, spoilage, and safety of food. Food mycology, as a coherent study, came of age with the appearance of major texts.[11,143,156] Initially, methods for studying fungi in foods were based on those developed in the medical microbiology laboratory, but now more appropriate techniques have been developed and validated at a number of international workshops on the standardization of methods for the mycological examination of foods.[155] It is especially important to use appropriate methodology that will distinguish between superficial contamination of surfaces with propagules such as spores and infection of the interior of a solid substrate with mycelium.

Traditionally mycologists have studied this group of eukaryotic organisms that are now considered to belong to at least two, possibly three, kingdoms. The majority of fungi, in the broadest sense, have in common a heterotrophic metabolism (which distinguishes them from plants and algae) and the presence of an outer wall (which distinguishes them from animals). However, what may now be referred to as the kingdom of "true fungi," which includes the zygomycetes, ascomycetes, basidiomycetes, and deuteromycetes, do not have a motile form at any stage in their life cycles. The oomycetes, which used to be grouped together with the zygomycetes as "phycomycetes," include many very important pathogens of food plants such as the potato blight caused by *Phytophthora infestans*. But, they do have a motile stage in their often complex life cycles, and their biology relates them more closely with some groups of algae now grouped in the kingdom Protista. What have been referred to above as the "true fungi" may well have evolved from the same group of organisms that gave rise to the chytridiomycetes, and it is now widely accepted that the latter should be included in the "true fungi."[2,167] However, the chytridiomycetes do not have a major impact on food mycology except that the group includes *Synchytrium endobioticum*, the causative agent of wart disease of the potato, and *Olpidium brassicae*, a parasite of cabbage roots that may act as a vector for several serious viral diseases. Another disease of brassicas, known as clubroot, is caused by *Plasmodiophora brassicae*, which belongs to yet another phylum with some affinities with the slime molds, also traditionally studied by mycologists.

These groups are summarized in Table 53–1, and even if this classification turns out not to reflect the real evolutionary relationships among the fungi *sensu lato*, the group referred to as the true fungi shows a trend in their biology that is important in their interactions with food. They have shown a steady evolution to increasingly terrestrial environments and to habitats of reduced water activity so that they are able to colonize solid substrates. This evolutionary process has led to the development of two lifestyles—the yeasts and the molds. Despite their apparent morphological simplicity, yeasts occupy niches in which a liquid phase is dominant and contain a rich source of nutrients, such as the nectaries of plants and the body fluids of insects and other animals. They show physiological adaptation to these environments, and many species are auxotrophic for one or more vitamins. Although the matter is still debated,[27] the biology of the yeasts suggests that they have evolved several times from different groups of filamentous fungi. The yeasts are not known to be associated with the production of mycotoxins, although they certainly are important in the spoilage and production of food.

The second lifestyle is the filamentous growth pattern based on the extension of the hyphal tip and the production of a complex branching anastomosing mycelium.[21,80] This mycelium is able to grow over, and penetrate into, complex solid substrates by the secretion of enzymes to degrade macromolecules and the absorption of the small molecules released. This growth form often is associated with the differentiation of specialized aerial hyphae that produce spores well adapted for air dispersal, the study of which forms an important part of air microbiology.[66] Efforts to control food spoilage by molds need to take account of the atmosphere as a persistent source of viable fungal propagules, and the ability of some species to grow at water activities as low as 0.61 (Table 53–2).[37,142]

The filamentous fungi, and their prokaryotic counterparts, the actinomycetes, also produce a diverse range of low molecular-weight compounds referred to as secondary metabolites, many of which are secreted into the environment.

The nature of secondary metabolism and the role of secondary metabolites in the biology of the producing organisms are still topics of active debate,[194] but many of these compounds do have biological activity, such as toxicity to microorganisms (antibiotics), plants (phytotoxins), and animals. When these mold metabolites are toxic to humans and domestic animals and are associated with food, animal feeds, or raw materials used for their production, they are known as mycotoxins.

There are also a number of fungi that produce macroscopic fruiting bodies, which have provided a source of food—mushrooms, boletes, and morels—in many parts of the world. Some species, such as *Amanita phalloides*, the deathcap, produce toxic metabolites in their fruit bodies and may cause illness, even death, if consumed.[129] Amanitoxin, produced by *A. phalloides*, causes irreversible liver and kidney damage with symptoms developing within 10 to 14 hours that may lead to death in 4 to 7 days if not diagnosed and treated. The chemically unrelated orellanin, produced by *Cortinarius orellanus*

Table 53–1 The Major Phyla of Organisms of Interest to the Food Mycologist

Kingdom	*Phylum*	*Examples*
Fungi	Chytridiomycota	*Synchytrium endobioticum* (Potato wart)
	Zygomycota	*Rhizopus stolonifer* (Fruit rots)
	Ascomycota*	*Penicillium*, *Aspergillus* (mycotoxins)
	Basidiomycota*	*Agaricus bisporus* (mushroom)
Protista	Oomycota	*Phytophthora infestans* (potato blight)
	Plasmodiophoromycota	*Plasmodiophora brassicae* (cabbage club root)

*Separation of these phyla is based on details of sexual reproduction. Many important species of molds and yeasts have no known sexual stage and are classified in form genera of the fungi imperfecti such as *Aspergillus* and *Penicillium*.

Table 53–2 Minimum Water Activities for the Growth of a Selection of Molds and Yeasts

Species	*Minimum* a_w
Rhizopus nigricans	0.94
Fusarium graminearum	0.89
Fusarium poae	0.89
Fusarium sporotrichioides	0.88
Alternaria alternata	0.88
Fusarium moniliforme	0.87
Cladosporium herbarum	0.85
Penicillium expansum	0.83
Penicillium aurantiogriseum	0.82
Penicillium verrucosum	0.81
Aspergillus flavus	0.81
Saccharomyces cerevisiae	0.80
Zygosaccharomyces bailii	0.80
Aspergillus versicolor	0.78
Aspergillus ochraceus	0.76
Eurotium chevalieri	0.71
Wallemia sebi	0.69
Zygosaccharomyces rouxii	0.65
Xeromyces (Monascus) bisporus	0.61

and related species, also causes irreversible kidney damage, signs of which appear after 3 to 14 days. Earlier symptoms including vomiting may occur within 4 to 9 hours of consumption, however. This species has been responsible for many deaths in parts of Eastern Europe. One of the more bizarre physiological effects is associated with coprine, produced by *Coprinus atramentarius*, which causes great discomfort when consumed with alcohol, a reaction that may be apparent within 10 to 15 minutes. However, because these toxins are a natural constituent of fruiting bodies deliberately ingested, usually as a result of mistaken identity, they are not included as mycotoxins, a distinction based on human biology rather than chemistry, biochemistry, or toxicology.

53.1.2 Mycotoxins in Food and Animal Feeds

Although a very diverse range of molds is able to produce toxic metabolites, and many of these may be important in animal husbandry, there are three genera that are especially important in foods: *Aspergillus*, *Penicillium*, and *Fusarium*. There are many textbooks on mycotoxins, mycotoxicoses, and mycotoxigenic fungi,[12,28,89,118,122,169,171] and the primary literature on these compounds is very extensive. In terms of acute toxicity (Table 53–3), the mycotoxins most commonly encountered in food are about 1 million times less toxic than the most lethal of the botulinum toxins and are significantly less toxic than many of the algal toxins. However, long-term chronic toxicity is of special concern because several of these mold metabolites are carcinogenic and influence the immune response of a number of animal species.

The occurrence of these toxins as contaminants of major components of diets (see Table 53–4) implies that exposure may occur over long periods of time. Although the number of incidents of acute poisoning of farm animals by mycotoxins is significant, the number of cases of confirmed acute mycotoxin poisoning in humans during the last few decades is small compared with the numbers of cases of food poisoning resulting from viruses and bacteria. In the Middle Ages, ergotism, from the production of alkaloids such as ergotamine (Figure 53–1) in cereals by the plant pathogen *Claviceps purpurea*, undoubtedly claimed many lives. At the beginning of this century, two major mycotoxicoses caused considerable suffering and mortality. They were alimentary toxic aleukia in Russia (ATA), a terrible illness caused by the consumption of cereals contaminated with trichothecenes such as T-2 toxin (Figure 53–2) produced by the plant saprotrophs *Fusarium sporotrichioides* and *F. poae*,[83] and yellow rice disease in Japan, associated with a number of toxigenic species of *Penicillium*, especially *P. islandicum*.[61] More recently, a tragic outbreak of aflatoxicosis caused by the consumption of corn contaminated with *Aspergillus flavus* was reported in India involving approximately 1,000 people, of whom nearly 100 died.[87] Undoubtedly, exposure to mycotoxins in the diet contributes to chronic ill health of poorer people in the humid tropics who depend on plant products that are inadequately stored after harvest. In more temperate parts of the world, mycotoxins are thought to cause the chronic kidney disease known as endemic nephropathy.[26]

Several very important mycotoxins, such as the sporidesmins, slaframine, and the tremorgens are associated with animal feeds and forages, but this review will not attempt to cover these although they will have an impact on the quality and quantity of meat and other animal products.[121] In two instances, the presence of mycotoxins in animal feed can lead to their presence in human foods because they pass through the food chain in either their original or metabolized form. These will be dealt with in the sections on aflatoxin and ochratoxin.

Many foods are susceptible to mold growth when stored under inappropriate conditions; a number of

Table 53–3 Toxicity and Biological Effects of Some Major Mycotoxins Found in Foods

Mycotoxin	*Biological Activity*	*LD_{50} (mg kg^{-1})*
Aflatoxin B_1	Hepatotoxic, carcinogenic	0.5 (dog), 9.0 (mouse)
Cyclopiazonic acid	Convulsions	36 (rat)
Deoxynivalenol	Vomiting, feed refusal	70 (mouse)
Fumonisin	Equine encephalomalacia Pulmonary edema in pigs Esophageal carcinoma	?
Ochratoxin	Nephrotoxic	20–30 (rat)
Patulin	Oedema, hemorrhage, possibly carcinogenic	35 (mouse)
Penitrem A	Tremorgen	1.05 (mouse)
Sterigmatocystin	Hepatotoxic, carcinogenic	166 (rat)
Tenuazonic acid	Convulsions, hemorrhage	81 (female mouse) 186 (male mouse)
Zearalenone	Estrogenic	not acutely toxic
Botulinum toxin B*	Blocks motor nervous system	10^{-6}
Aeruginosin†	Hepatotoxin	0.05

**Clostridium botulinum*
† Microcystis aeruginosa

foods have evolved in which mold growth is an essential part of their manufacture (eg, mold-ripened cheeses and sausages, tempeh, and miso). Mycotoxins may be formed in foods, raw materials for food production, or animal feeds, and the production of each mycotoxin usually is limited to a small range of fungal species or even strains within a species. Many mycotoxigenic species are able to produce several mycotoxins, and foods may be contaminated with several species of mold. The biosynthetic pathways leading to the production of mycotoxins are diverse and generally involve either polyketide, mevalonate, or amino acid precursors and mixtures from these three pools of intermediates.[120,173] It is likely, therefore, that contaminated foods will contain a cocktail of toxins that can interact synergistically. For example, Kanisawa[86] described a synergistic effect of citrinin on the carcinogenic activity of ochratoxin A in mice, and Sato and Ueno[157] provided convincing evidence of the synergistic interaction of ochratoxin A and T-2 toxin on their acute toxicity in day-old chickens. Indeed, it is probable that the outbreak of Turkey X disease in 1959, which led to the discovery of the aflatoxins, was really the result of the synergistic interaction between cyclopiazonic acid and aflatoxin.

Although it is essential to recognize the potential for these complex interactions, it is simpler to deal with individual toxins. The following sections will deal with the major mycotoxins in human foods.

53.2 THE AFLATOXINS

53.2.1 Production

Aflatoxin B_1 (Figure 53–3) is the most important of this large family of compounds and is produced by three closely related species: *Aspergillus flavus*, *A. parasiticus*, and the more recently described *A. nomius*.[93] Strains of *A. parasiticus* and *A. nomius*, but not *A. flavus*, also are able to produce aflatoxin G_1 (Figure 53–3). The aflatoxins arise essentially

Table 53–4 Some Major Mycotoxins Found in Foods

Mycotoxin	*Major Foods*	*Common Producing Species*
Aflatoxins	Corn, groundnuts, figs, tree nuts	*Aspergillus flavus* *A. parasiticus*
Aflatoxin M_1	Milk, milk products	(secreted by cow after metabolism of Afl B_1)
Cyclopiazonic acid	Cheese, corn, groundnuts, Kodo millet	*Aspergillus flavus* *Penicillium aurantiogriseum*
Deoxynivalenol	Cereals	*Fusarium graminearum* *F. culmorum*
Fumonisin	Corn	*Fusarium moniliforme*
Ochratoxin	Corn, cereals, coffee beans	*Penicillium verrucosum* *Aspergillus ochraceus*
Patulin	Apple juice	*Penicillium expansum*
Penitrem A	Walnuts	*Penicillium aurantiogriseum*
Sterigmatocystin	Cereals, coffee, beans, cheese	*Aspergillus versicolor*
Tenuazonic acid	Tomato paste	*Alternaria tenuis*
Zearalenone	Corn, barley, wheat	*Fusarium graminearum*

from a decaketide precursor that has undergone extensive rearrangement. The molecular biology of their biosynthesis has been reviewed.[10] *A. parasiticus* is the most toxigenic of the three species, with perhaps 80% to 90% of isolates being able to produce aflatoxins. In the case of *A. flavus*, perhaps only 30% to 40% of isolates on a worldwide basis are aflatoxigenic, but this varies from one part of the world to another and also may be dependent on the substrate from which the isolates were obtained. Some strains of this species have been used in the production of koji and *A. oryzae*, which is most frequently used for koji production and is considered to have evolved from *A. flavus* by continuous industrial use.[44] Both *A. flavus* and *A. nomius*, but not *A. parasiticus*, also produce cyclopiazonic

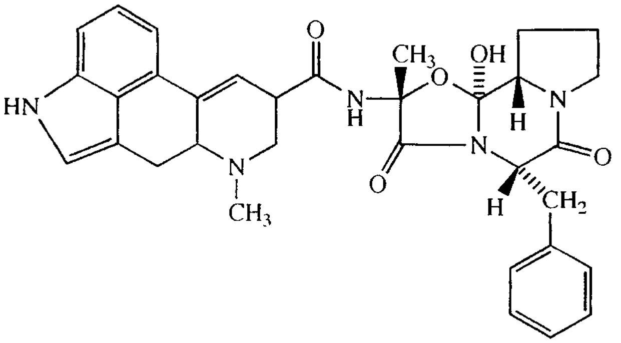


Figure 53–1 Ergotamine

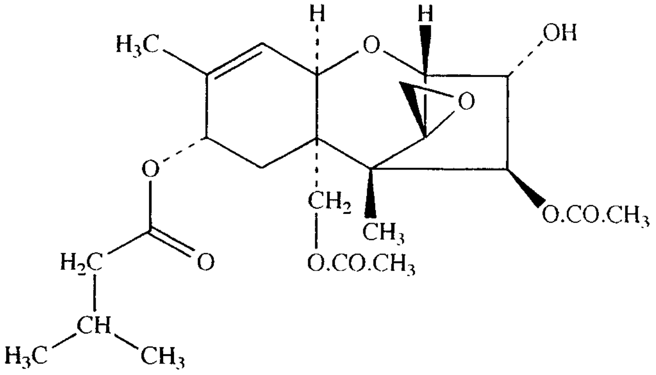
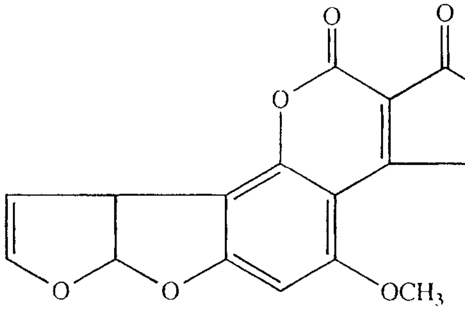
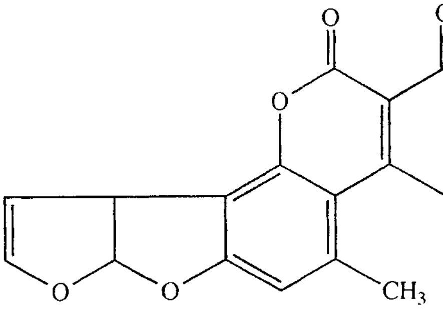


Figure 53–2 T-2 Toxin



Aflatoxin B_1



Aflatoxin G_1

Figure 53–3 Aflatoxins B_1 and G_1

acid that may act synergistically to influence the apparent toxicity of aflatoxin. Gallagher and colleagues[58] studied a collection of 54 isolates of *A. flavus* and found that 14 produced cyclopiazonic acid and aflatoxin concurrently, 4 produced only aflatoxin, and 14 produced only cyclopiazonic acid. Thirty-three percent of the strains of *A. flavus* were aflatoxigenic under the conditions used. Although the older literature contains references to other species even belonging to different genera producing aflatoxins, these have never been confirmed with the exception of a report[98] claiming to confirm an earlier observation that aflatoxins are produced by *Aspergillus ruber*.[92]

53.2.2 Occurrence

The aflatoxigenic molds occur in warmer parts of the world, and aflatoxins may be produced in a wide range of tropical and subtropical food commodities, such as figs, tree nuts, and cereals. The most important crops are corn and groundnuts, but it can also occur in temperate crops such as wheat.[166] Table 53–5 lists some of the more recent reports on the occurrence of aflatoxins in foods.

Although the aflatoxigenic species of *Aspergillus* cannot normally compete with the indigenous mold flora in temperate climates, viable spores occur in the atmosphere and on imported commodities in many parts of the world, and there have been occasions when aflatoxin production has occurred in British and Scandinavian barley. The storage of high-moisture cereals for nutritious winter feeds can be achieved by lactic fermentation or by adding acids such as ethanoic and propionic acids. The presence of 1% propionic acid completely inhibits the production of aflatoxin and ochratoxin in high-moisture cereals;[192] however, in both the United Kingdom (UK)[68] and Sweden,[140] aflatoxin was found in home-grown cereals inadequately treated with propionic acid. Indeed, it was shown subsequently that certain concentrations of propionic acid, which only partially inhibit growth of *A. flavus*, actually enhance the levels of aflatoxin produced.[3]

The complex ecology of these molds recently was reviewed by Cotty et al.[38] Although the production of aflatoxin initially was considered to be a problem in postharvest crops stored at inappropriate temperatures and water activities, it is now well appreciated that these compounds can be present in the field before harvest. *Aspergillus flavus* and *A. parasiticus* may infect healthy plants at a very early stage in their development and establish a benign endophytic relationship in crops such as corn and groundnuts. However, if the infected plant is stressed by such conditions as drought, then significant levels of aflatoxins may be produced in apparently healthy seed and are thus present postharvest, however good the subsequent storage conditions. This group of fungi also has an association with insects and insect damage of developing crops may also predispose them to infection and aflatoxin contamination.

Contamination of components of a crop, such as individual corn kernels or groundnuts, has a very skewed distribution; for example, the maize kernels on the same cob can be contaminated with a wide range of concentrations of aflatoxins, some having undetectable levels even though infected with *A. flavus*.[97] Individual cotton bolls on the same stem may have as little as 10 μg aflatoxin kg^{-1} or as much as 22,240 μg kg^{-1}, and again many of the bolls remain uncontaminated.[39] A detailed study of a large consignment of dried figs dramatically demonstrated the extremely skewed distribution of aflatoxin,[165] and an analysis of the distribution of aflatoxin in pistachios demonstrated that 90% of the aflatoxin was

Table 53–5 Recent Reports on the Occurrence of Aflatoxins in Foods

Commodity	*Country*	*Levels*	*Reference*
Groundnuts	India	21% >30 ppb	13
Corn (field)	Argentina	19.6% +ve	146
Corn	Brazil	12.3% 10 to 900 ppb	153
Corn	India	9–40% >20 ppb	168
Corn	Pakistan	48 × 500 g 21 to 385 ppb (distribution study)	81
Mustard seed	India	44% Tr to 750 ppb	154
Mustard cake	India	80% 100 to 1420 ppb	154
Mustard oil	India	33% 55 to 87 ppb	154
Dried figs	Turkey	4% mean 112 ppb	16
Herbs and spices	UK	6% >10 ppb	105
Spices	Egypt	4% 10 to 35 ppb	7
Meat products	Egypt	3.3% 6 to 175 ppb	7

present in 4.6% of the product, which was demonstrably of low quality.[158] This latter study demonstrated that it should be possible to reduce total aflatoxin in a product by identifying and rejecting the contaminated nuts. The significance of these studies is of considerable importance when establishing sampling plans for monitoring compliance with the legislative or advisory limits discussed below.

53.2.3 Biological Effects

The aflatoxins are acute hepatotoxins, and the four most frequently encountered members of the family vary in their toxicity to a single animal species (Table 53–6). Aflatoxins also are known to be carcinogenic in some animal species,[104] such as the rat and the rainbow trout. The presence of 100 μg kg^{-1} in a diet fed to rats for 54 to 88 weeks gave 100% incidence of tumors, and as little as 8 μg kg^{-1} in a diet fed to rainbow trout for a year gave 40% incidence of tumors. Liver cancer in rainbow trout became a problem when fish farms changed from animal protein to cottonseed meal as a source of protein in the pelleted feed. Rainbow trout are so sensitive that the etiology of this disease was unresolved until after the discovery of the aflatoxins following the outbreak of Turkey X disease in the UK in 1959 and the development of sufficiently sensitive and specific analytical methods. Carcinogenicity is species specific and no tumors were reported in mice fed on a diet containing as much as 150 μg aflatoxin kg^{-1} for 80 weeks. Thus there is considerable variation in both the acute and chronic toxicity of a single aflatoxin to a range of animal species and between the sexes and ages of the same species (Table 53–7). These observations would suggest that the toxicological effects of the aflatoxins are influenced by, or even dependent on, the metabolism of aflatoxin after intake into the body. This is now known to be the case.[124] The most important products of animal metabolism are shown in Figure 53–4. Aflatoxin B_1 epoxide certainly plays a central role in the toxicology of the aflatoxins,[71] and it has been shown to react very effectively and specifically with N^7 of guanine in DNA to form well-defined adducts. Aflatoxin B_1 is certainly acutely toxic to humans, is probably responsible for liver necrosis following chronic exposure, and may be involved in the epidemiology of human liver cancer in some parts of the world.

Table 53–6 Single Dose LD_{50} of Aflatoxins in Day-Old Ducklings

Aflatoxin	*LD_{50} (mg kg^{-1})*
B_1	0.36
B_2	1.9
G_1	0.78
G_2	3.5

Where hepatocellular carcinoma in humans is high, the presence of aflatoxin is accompanied by exposure to hepatitis B virus. Studies in Gambia supported a hypothesis that liver damage arising from hepatitis B infection may enhance the carcinogenic activity of aflatoxin.[198] However, detailed studies in Taiwan suggested that the role of aflatoxin in the epidemiology of liver cancer is not dependent on infection by hepatitis B virus.[74]

There are several parts of the world, especially in the tropical and subtropical areas of Africa, India, Southeast Asia, and Central and South America, where many people are exposed to foods contaminated with aflatoxin.[177] The toxicological response may be acute, as in the case of the outbreak of aflatoxicosis in India,[87] or chronic leading to liver damage and possibly eventually to primary hepatocellular carcinoma. The evidence for a strong statistical association between aflatoxin injestion and primary liver carcinoma is based on more than 25 years of epidemiological surveys in several parts of the world.[200] It is certain that the association of liver cancer and aflatoxin is complex and may involve synergistic interactions with other agents such as hepatitis B virus.[4,78,164,191] It has been shown that, not only does aflatoxin react with guanine residues in DNA after activation to the epoxide, but that there are very specific hot spots for such reactions.[75,102,183] Both hepatitis B virus and aflatoxin may enhance mutations of the p53 tumor suppressor gene;[77] thus each may contribute independently as well as acting synergistically to the incidence of hepatocellular carcinoma.

Table 53–7 LD_{50} Values for Aflatoxin B_1

Animal Species	*Oral LD_{50} (mg kg^{-1})*
Cat	0.55
Dog	0.5–1.0
Guinea pig	1.0–1.4
Hamster	10.2
Rat (1 day old)	0.56
Weanling rat (male)	5.5
Weanling rat (female)	7.4
Rat (adult male)	7.2
Rat (adult female)	17.9
Mouse	9
Rabbit	0.3
Duckling (1 day old)	0.34
Chicken	6.3
Pig	0.6
Rainbow trout	0.8
Sheep	1.0–2.0
Baboon	2.0
Macaque monkey	7.8

One aspect of the metabolism of aflatoxin, which is especially important in assessing and controlling human exposure, is the conversion of aflatoxin B_1 to its 4-hydroxy derivative, aflatoxin M_1 (Figure 53–4) in the cow[76] and its subsequent excretion in milk. The carryover of aflatoxin B_1 in feedstuffs as aflatoxin M_1 in milk is of the order of 1% to 2% but varies from animal to animal and day to day, being strongly influenced by the physiological status of the cow. The occurrence, toxicity, and regulation of aflatoxin M_1 have been thoroughly reviewed by Van Egmond.[188] Although it is similar in acute toxicity to aflatoxin B_1, the milk metabolite is significantly less carcinogenic in experimental animals.

This same metabolic transformation also occurs in humans, and an extensive study in the United Arab Emirates demonstrated the presence of aflatoxin M_1 in human milk and showed that many commonly used food ingredients, such as cereals, pulses, pistachios, Brazil nuts, walnuts, and almonds, contained aflatoxin B_1.[151,152]

There are now many reports of the occurrence of aflatoxins and aflatoxin derivatives in human blood, urine, and milk. Based on a detailed study in the Philippines during 1967–1969, Campbell et al.[22] demonstrated the presence of aflatoxin M_1 in the urine of children exposed to dietary aflatoxin B_1. The main source in this case was probably peanut butter, 29 samples of which had a mean concentration of 500 ppb. From these studies the authors suggested that aflatoxin M_1 could be detected in the urine of children if they were consuming in excess of 10 to 15 μg aflatoxin B_1 per day. Aflatoxin B_1 itself was not detected in the urine samples and the efficiency of conversion of dietary B_1 to urinary M_1 was only a few percent. This was confirmed in a study in Guangzxi Province, China, in which a mean daily intake of B_1 and mean daily excretion of M_1 were 66 and 0.7 μg per person per day respectively.[206]

Aflatoxin M_1 was detected in the breast milk of Sudanese mothers[40] and in the blood and urine of

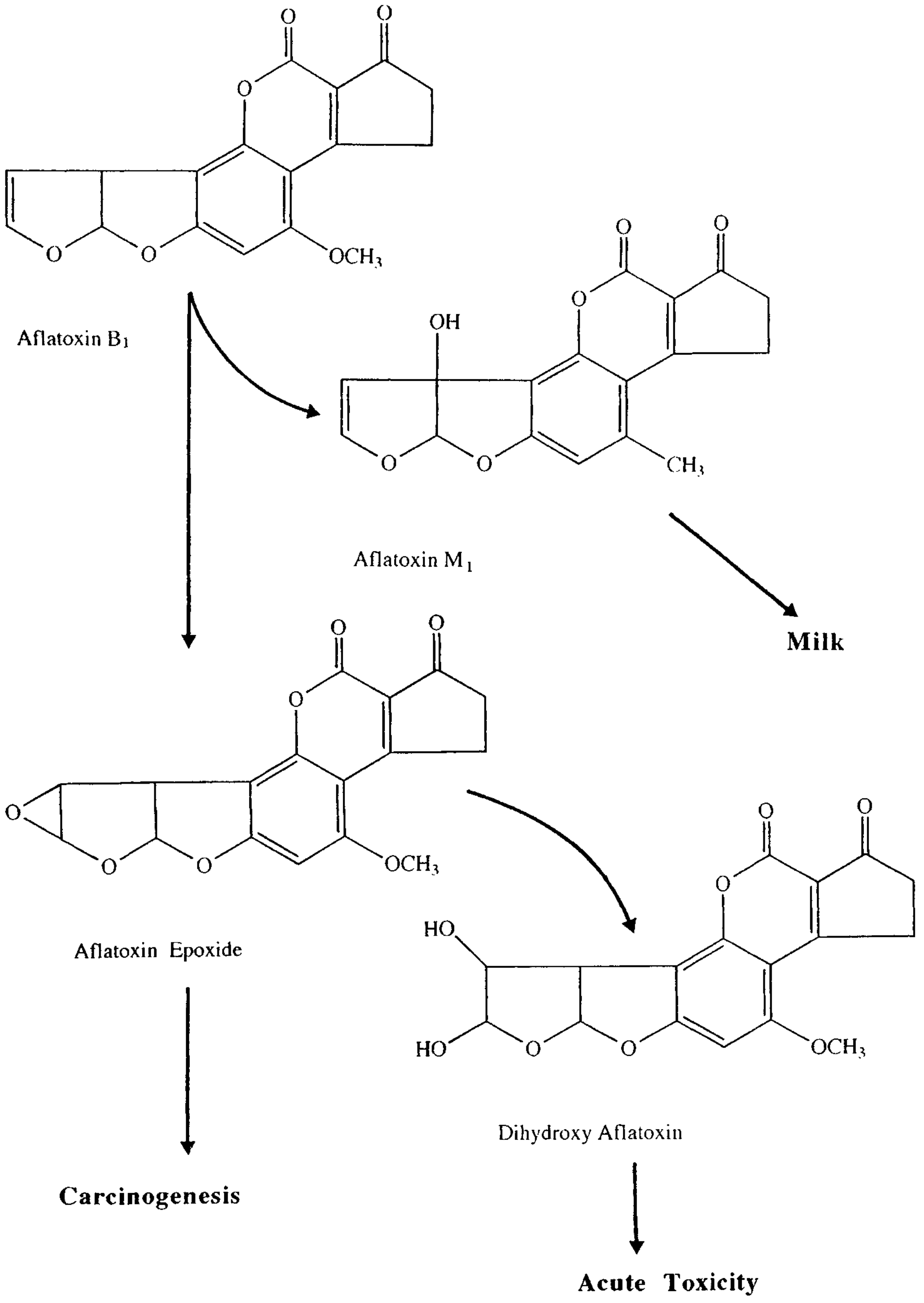


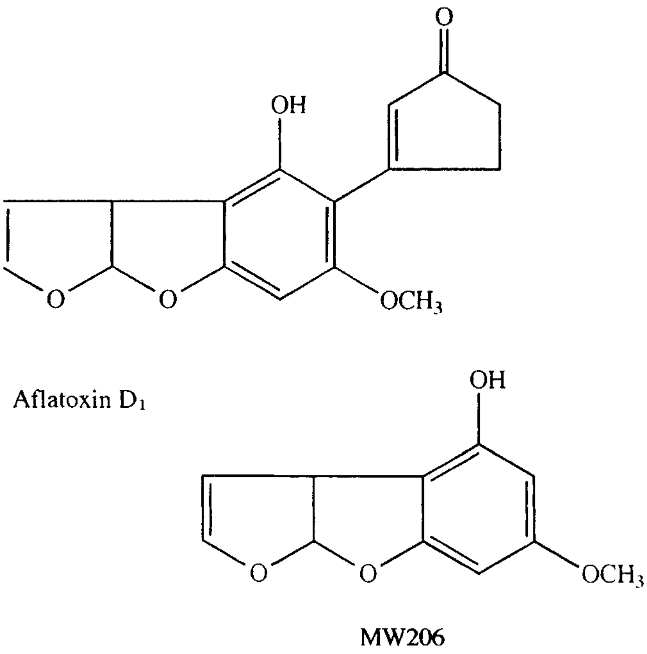
Figure 53–4 The Major Products of the Metabolism of Aflatoxin B_1 in the Liver.

malnourished children in Sudan.[41] There are also reports of aflatoxin M_1 in human milk samples from rural villages in Zimbabwe[199] and Ghana.[95] As part of the latter study extended to Nigeria, venous blood from mothers and cord blood samples from infants were also studied, and aflatoxins were found in a significant number of these samples.

53.2.4 Control

Because aflatoxins are potentially widespread in occurrence and have an insidious combination of acute and chronic toxicity, it is prudent to control their presence in foods. Many countries have legislation establishing maximum tolerated levels,[189] which will be discussed later. Because there may be circumstances in which it is difficult to avoid contamination of large, economically valuable consignments of food and feed commodities with aflatoxins, a great deal of effort has been put into methods for decontamination using both physical[196] and chemical methods.[137] Although many chemical reagents have been tried, the most promising on a large scale is treatment with ammonia.[131] The major breakdown products from aflatoxin B_1 are aflatoxin D_1 and a compound having a molecular weight of 206 (Figure 53–5).

Although the initial opening of the δ-lactone ring is reversible, once this compound has undergone decarboxylation to aflatoxin D_1 and subsequent loss of the cyclopentenone ring to MW_{206}, the process is irreversible and the products are very much less toxic than aflatoxin B_1. One matter of concern was that degraded products from ammoniation of aflatoxin-contaminated commodities might be reconverted to active forms of aflatoxin in the acidic environment of the stomach. This has been shown not to be the case,[195] and a detailed inventory of the degradation of aflatoxin B_1 has been performed using ^{14}C-labeled material.[113] Reduction in levels of aflatoxin can be very dramatic; thus it has been shown that naturally contaminated cottonseed meal containing 4,000 ppb aflatoxins contained only 4 ppb after treatment for 30 minutes with 4% ammonia at 2.76 bar (40 psi) and 100° C.[130] In a detailed study in France, groundnut oil cake, contaminated with aflatoxins (1,000 ppb B_1 and 170 ppb G_1), was treated with ammonia gas for 15 min at 95° C at a pressure of either 2 or 3 bar. The two treatments led to a residual contamination of 140 ppb B_1 + 20 ppb G_1 and 60 ppb B_1 + 10 ppb G_1 respectively and were used in an 18-month feeding trial with rats.[54] It was reported that the percentage of hepatic tumors was high in animals fed untreated cake, fell sharply with the medium treatment, and was zero for the cake treated at 3 bar.



Aflatoxin D_1

MW206

Figure 53–5 Degradation Products of Aflatoxin B_1 Treated with Ammonia

For particulate commodities, such as nuts, in which it is known that the distribution of contamination is very skewed, it may be possible to use technologically sophisticated equipment to sort and discard the contaminated units. Such a system may involve an electro-optical sorter to remove fluorescent nuts when the fluorescence is known to be associated with aflatoxin contamination.[53]

It may also be possible to control the production of aflatoxin in the field by an integrated program of agricultural management that may include plant breeding, improved irrigation, and replacement of aflatoxigenic strains by non-aflatoxigenic strains of *A. flavus*.

53.3 THE OCHRATOXINS

53.3.1 Production

Ochratoxin A ($C_{20}H_{18}ClNO$) is a phenylalanyl derivative of a substituted isocoumarin (Figure 53–6), produced by *Penicillium verrucosum* in temperate climates and by several species of *Aspergillus* in warmer and tropical parts of the world. The most important species of *Aspergillus*-producing ochratoxin A is *A. ochraceus*; although *A. sclerotiorum*, *A. sulphureus*, and *A. melleus* also produce ochratoxin, they are rarely isolated from foods.

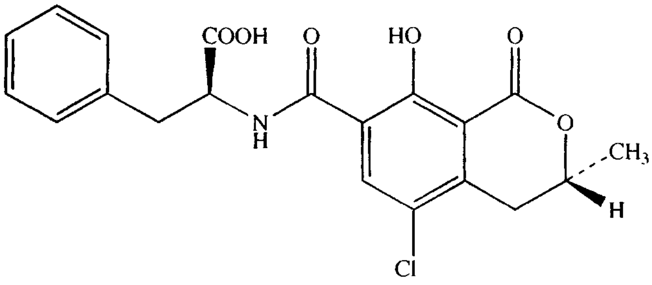


Figure 53–6 Ochratoxin A

Petromyces alliaceus, isolated from onions, is a good ochratoxin producer in the laboratory, and two species related to *A. niger*—*A. citricus* and *A. fonsecaeus*—have also recently been shown to produce ochratoxin, but their significance in foods is not yet known (Frisvad, personal communication).

Until relatively recently, ochratoxin A was considered to be produced by several species of *Penicillium*, such as *P. palitans*, *P. commune*, *P. variabile*, *P. purpurescens*, *P. cyclopium*, and *P. chrysogenum*, and *P. viridicatum* has been especially associated with this mycotoxin in the earlier literature. Ciegler et al.[34] studied 52 isolates of molds identified at that time as *P. viridicatum* and divided them into three groups on the basis of growth rate, colony morphology, odor, source, and ability to produce ochratoxin and/or citrinin. Group I (21 isolates) did not produce ochratoxin or citrinin and represent what is now accepted as the well-defined species *P. viridicatum*. Groups II (17 isolates) and III (13 isolates) included strains that produce either ochratoxin or citrinin or both and would now be referred to *P. verrucosum*.

In their detailed studies of secondary metabolite production by species of the terverticillate penicillia, Frisvad also demonstrated that strains previously referred to as *P. viridicatum* could be separated into a number of distinct chemotypes among which was a group producing ochratoxin A.[55] This group subsequently was shown to fit the description of *P. verrucossum*,[56,141] and it is now widely accepted that this is the only species of *Penicillium* producing ochratoxin A. There are also several unconfirmed but important reports of other species of *Aspergillus* and *Penicillium* producing ochratoxin.[29,103]

The different requirements of the two major ochratoxin-producing species are further illustrated by the influence of different plant commodities when used as the substrate for growth and ochratoxin production. Thus Madhyastha et al.[106] clearly showed that *Aspergillus ochraceus* (= *A. alutaceus*) produces more ochratoxins on groundnuts and soybeans than on corn and wheat, whereas the reverse was true for *P. verrucosum*. At an even more subtle level of substrate mold interaction, Häggblom and Ghosh[70] showed that the postharvest production of ochratoxin A by both *A. ochraceus* and *P. verrucosum* on barley was influenced by the level of nitrogen fertilizer applied to the growing crop. Fertilizer applications of 0, 90, or 240 kg ha^{-1} produced grain containing 9.1%, 10.4%, or 12.0% protein, respectively, and ochratoxin production increased with increasing protein content in a manner that could not be explained simply by the effects of the substrates on fungal growth.

The two major ochratoxin-producing molds differ in the conditions of temperature and water activity (a_w) that are optimum for growth and ochratoxin production.[8,128] Thus, the minimum a_w for growth of *P. verrucosum* is 0.81 and for *A. ochraceus*, 0.76, and at optimum a_w the temperature range for ochratoxin is 4° C to 31° C (opt 24° C) for *P. verrucosum* and 12° C to 37° C (opt 25° C) for *A. ochraceus*.

Biosynthetically, the isocoumarin structure of the ochratoxins is based on a pentaketide skeleton, to which a chlorine atom has been introduced and a C_1 unit has been added and subsequently oxidized to carboxyl. It is through this additional carboxyl group that L-phenylalanine is linked. This same pentaketide skeleton also can give rise to the mycotoxins austdiol (by the addition of two C_1 units) and citrinin (by the addition of three C_1 units), the C_1 units being derived from methionine.[119]

Like most mycotoxins, and other secondary metabolites, ochratoxin A is one of a family of compounds (Figure 53–6), but it is usually only ochratoxin A and occasionally the dechloro compound ochratoxin B that are found to occur naturally in moldy plant products. It is possible that ochratoxin C, the ethyl ester of ochratoxin A, is more widespread than presently recorded. Its toxicity is comparable to that of ochratoxin A, because it is readily de-esterified in the animal body, whereas the absence of the chlorine atom in ochratoxin B is associated with a considerable decrease in toxicity.

As already noted in the case of aflatoxin, the secondary metabolism of molds also may be influenced by the presence of inhibitory compounds such as agricultural biocides.[123] Ochratoxin biosynthesis is no exception and it has been shown, for example, that the presence of subinhibitory concentrations of phosphine may increase levels of ochratoxin produced.[193] On the other hand, dichlorvos inhibit ochratoxin biosynthesis at concentrations that have relatively little effect on growth.[201] Ionizing radiation, at levels that damage spores but do not kill them, may lead to enhanced levels of ochratoxin production,[5,134] but the significance of these observations for irradiated foods has still to be critically assessed.

53.3.2 Occurrence

P. verrucosum is especially associated with stored cereals, although it has also been isolated from meat and fish;[127] however, the occurrence of ochratoxin A in meat products is usually due to transmission into muscle, kidney, and blood in animals fed on contaminated animal feed such as barley. Ochratoxin A may also be transferred from dietary intake into milk because it has been detected in both cow's milk and human milk.[18] This is the second example of a mycotoxin that can be passed through the food chain and appear in a commodity that has not been molded. *A. ochraceus*, on the other hand, is common on coffee beans and spices and also is isolated from cocoa beans, soybeans, groundnuts, rice, and corn.[91] Krogh[90] has provided an extensive list and bibliography of foods and feeds of plant origin in which ochratoxin A has been found to occur naturally, and reports of its occurrence in food commodities continue to appear (Table 53–8). The demonstration that ochratoxin is not destroyed by roasting of naturally contaminated coffee beans[178,185] has led to an increase in the surveillance of this particular commodity and the demonstration of a persistent but low level of contamination (Table 53–9). Earlier reports of degradation during roasting often were based on materials spiked with pure toxin, and this superficial contamination is rather easily removed with the outer layers of the coffee beans during the roasting process.

53.3.3 Biological Activity

Ochratoxin has been known for some time to be a major etiological agent in porcine nephropathy,[88] and by analogy it has been considered to be associated with the chronic progressive kidney disease in humans known as Balkan endemic nephropathy.[24] Nearly 20 years ago, there were suggestions that the human disorder may involve mycotoxins other than ochratoxin,[9] and more recently Mantle et al.[111] have provided convincing evidence that toxic metabolites of penicillia that do not produce ochratoxin may be important in Balkan nephropathy. Nevertheless, concern about ochratoxin A has been highlighted by the increasing evidence that it should be considered as a carcinogen with genotoxic properties as well as being a potent nephrotoxin. The availability of improved methods of analysis has demonstrated that ochratoxin is quite widespread in foods, and its presence in human body fluids confirms that there is a significant exposure within the human population.[18]

Ochratoxin A is immunosuppressive[96] and inhibits protein biosynthesis. It almost certainly has a number of interacting effects on target organs, but at the molecular level it is known to inhibit the

Table 53–8 Reports of the Occurrence of Ochratoxin in Foods

Commodity	*Country*	*Levels*	*Reference*
Corn	France	2% 15 to 200 ppb	59
Corn	Yugoslavia	8.3% 6 to 140 ppb	136
Corn	UK	13% 1 to 11.2 ppb	108
Wheat	Yugoslavia	8.2% 14 to 135 ppb	136
Wheat	Denmark	29% 0.05 to 51 ppb	84
Wheat	Czechoslovakia	12% >20 ppb	57
Barley	Yugoslavia	12.5% 14 to 27 ppb	136
Barley	Denmark	27% 0.05 to 5.6 ppb	84
Barley	Czechoslovakia	6% >20 ppb	57
Barley	UK	17% 1 to 45 ppb	108
Soya	UK	26% 50 to 500 ppb	107
Cocoa beans (raw)	UK	18% 100 to 500 ppb	107
Cocoa (roasted)	UK	16% 100 ppb	107
Herbal drugs	India	40% 0.07 to 2.3 ppm	150
Pig kidney	UK	15% 1 to 9.3 ppb	108
Black pudding	UK	13% 1 to 1.8 ppb	108

Source: Data from P. Krough, Ochratoxin in Food, in *Mycotoxins in Food*, P. Krough, ed., pp. 97–121, © 1987, Academic Press.

Table 53–9 Reported Occurrence of Ochratoxin A in Coffee

Commodity	*+ve/total (%)*	*Range (ppb)*	*Reference*
Green beans	3/68 (4.4)	20 to 80	100
Green beans	9/40 (22.5)	0.5 to 23	23
Green beans	4/22 (18.2)	9.8 to 46	184
Beans	5/68 (7.4)	3.2 to 17	186
Beans	16/29 (55)	0.2 to 15	115
Green beans	13/25 (52)	1.2 to 56	178
Coffee brews	18/40 (45)	0.4 to 7.8	178
Soluble coffee	64/80 (80)	0.1 to 8	135
Roast and ground	17/20 (85)	0.2 to 2.1	135
Green (robusta)	55/75 (73)	0.26 to 27	110
Green (arabica)	31/153 (20)	0.26 to 9	110
Green (decaf.)	6/17 (35)	0.26 to 7.7	110

phenylalanyl transfer RNA synthetase enzyme involved in protein biosynthesis. It is thus natural to ask whether other amino acid analogs would have analogous effects. A number of other analogs have been synthesized and shown to be active to some extent in *in vitro* tests with monkey kidney epithelial cells.[174] Creppy et al.[43] used eight such analogs in a cell-free system based on the appropriate purified tRNA synthetases from a yeast and demonstrated that they all had some inhibitory activity but that it varied with the amino acid. Ochratoxin A itself (the phenylalanyl derivative) was the most active, and the tyrosinyl, valyl, seryl, and alanyl derivatives were very active, but the prolyl derivative was the least active. Several natural analogs of ochratoxin A have been shown to occur in cultures of *Aspergillus ochraceus*, in which the phenylalanyl side chain is replaced with either serine, hydroxyproline, or lysine,[69] and further analogs of ochratoxin A have been chemically synthesized for the study of metabolic and immunological structure/activity relationships.[202]

Ochratoxin A has been classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen.[79] There is no doubt that doses as low as 70 μg kg^{-1} body weight can induce kidney tumors in male rats. The male rat is about 10 times more sensitive than the female rat and about 100 times more sensitive than the male mouse to the carcinogenicity of ochratoxin A. These observations do not in themselves help us assess whether ochratoxin is carcinogenic to humans, but it is prudent to maintain vigilance until it is confirmed that it is not. Many tests for genotoxicity have proved to be negative but the occurrence of single-strand breaks in DNA in a number of tissues in mice exposed to ochratoxin A have been demonstrated.[42] Evidence for the role of metabolic activation involving cytochrome P450 in the mutagenicity and genotoxicity of ochratoxin A also has been presented;[47] and this article should be consulted for a discussion of the recent literature on this subject. Risk assessments of ochratoxin A have led to recommendations of provisional tolerable daily intakes of between 5 and 16 ng kg^{-1} body weight (bw) per day. There are two ways of estimating exposure: analysis of components of diet or analysis of body fluids, such as blood. Both of these methods have been used in a number of European countries that are in reasonable agreement, suggesting mean exposures of the order of 1 to 2 ng kg^{-1} bw per day, the greatest exposure being from cereals and cereal products and only about 0.06 ng kg^{-1} bw per day from coffee and coffee products.

53.3.4 Control

Once ochratoxin A has been formed in a food, it would seem likely that it would be difficult to remove by most forms of food processing. The ability to survive the coffee roasting process has already been discussed above and it has been shown more recently that, even at temperatures as high as 250° C, complete destruction of ochratoxin A was not achieved.[15] The calculated half-lives for ochratoxin A in dry wheat were 707, 201, 12, and 6 minutes at 100° C, 150° C, 200° C, and 250° C, respectively, and in wet wheat the corresponding figures were 145, 60, and 19 minutes at 100° C, 150° C, and 200° C. Cooking, with or without previous soaking, removed a significant amount of ochratoxin from beans but did not lead to total destruction. Beans still contained 16% to 60% of the initial ochratoxin contamination and it seems probable that the material removed was leached out of the beans rather than destroyed.

53.4 PATULIN

53.4.1 Production

Patulin (Figure 53–7) is a toxic and antibiotic metabolite produced by several species of molds in the genera *Penicillium*, *Aspergillus*, and *Paecilomyces*, but the most important in the context of human food production is *Penicillium expansum*, a soft rot pathogen of apples and pears. *Aspergillus clavatus* commonly is associated with malted barley residues used in animal feeds and was implicated in an outbreak of patulin poisoning of cattle that grazed on barley stubble. Moreau provides a detailed account of the role of this species in outbreaks of poisoning of cattle fed on barley malt and cereal sprouts.[118] *Paecilomyces*, the imperfect stage of *Byssochlamys*, may contaminate silage and is implicated in the occasional problem with patulin contamination in silage. Because patulin first was isolated independently by several groups as a potentially useful wide-spectrum antibiotic, it has been known under several different names: clavacin, clavatin, claviformin, expansin, leucopin, mycoin C, penicidin, and tercinin. Indeed, it underwent quite extensive trials as an antibiotic before being abandoned on toxicological grounds.

Patulin proved to be a very useful model for studies of the biosynthesis of polyketide secondary metabolites, being essentially an extensively rearranged tetraketide,[205] but current interest in this molecule arises because of the toxicological significance of its presence in foods.

53.4.2 Occurrence

Possibly the first report of *Penicillium expansum* producing patulin in apples was that of Brian, Elson, and Lowe,[19] and the first report of its natural occurrence in apple juice was that of Scott et al.[162] Since then, it has been found in a range of foods based on fruits from many countries in concentrations ranging from 1 to 250,000 μg kg^{-1}.[145] Concern over patulin in the UK was expressed when a survey of samples of apple juices in 1992 demonstrated the presence of unexpectedly high concentration in 4 of 32 samples.[108] In the same year, an Australian survey showed that 57.5% of fruit juices tested contained patulin, 28% of which exceeded 50 μg l^{-1}.[20] The presence of patulin in a fruit juice is a useful indicator that the juice was expressed from poor quality fruit, which is undesirable and should be avoidable with good manufacturing practice; however, increasingly detailed studies of the occurrence of patulin in apples has demonstrated that it may occur in what looks like sound fruit. This is particularly a problem with those varieties that have an open core structure, such as Bramleys, in which mold growth can occur in the core. The presence of patulin in apple juice usually would arise from contaminated apples but, in the laboratory, *P. expansum* can produce patulin when growing in apple juice. In this situation, it has been shown that there may be quite a narrow pH range (3.2 to 3.8) for optimum patulin production.[46]

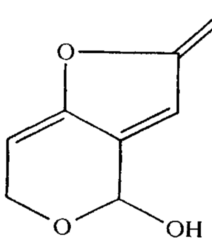


Figure 53–7 Patulin

53.4.3 Biological Activity

Patulin has an acute oral LD_{50} in rodents of about 30 to 50 mg kg^{-1} [197] and has been shown to be teratogenic, immunotoxic, and neurotoxic and to cause gastrointestinal disturbances in rats. Patulin is quite rapidly excreted from an animal, and a single dose of ^{14}C-labeled patulin was excreted in the feces (49%), urine (36%), and as $^{14}CO_2$ in expired air (1% to 2%) within 7 days.[45]

A review of the toxicology of patulin has made it prudent to regard it as a mutagen, although none of the Ames type of assays were positive in the pres-

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ence or absence of exogenous activating systems. The evidence for carcinogenicity still is equivocal; however, because of all these observations, and an increased popularity of apple juice, an advisory level of 50 µg l^{-1} has been established in the UK, and a survey carried out in 1993 showed that only 3 of 62 samples exceeded that level and none of these were in excess of 100 µg l^{-1}. A further survey reported in 1995[109] showed no detectable levels of patulin in any of 128 samples of fruit juice or 26 samples of tomato products.

53.4.4 Control

In apples molded by *Penicillium expansum*, most of the patulin is confined to the region of damaged tissue, and simply removing the lesion reduces the toxin by 90%, but if 1 cm around the lesion is also removed, no patulin is detectable in the rest of the apple.[181]

Although stable in some aqueous environments at low pH, patulin is chemically reactive, and it has been known for some time that it disappears during a yeast fermentation.[175,176] Patulin has wide-spectrum antibiotic activity and it has been demonstrated that up to 50 mg l^{-1} (a concentration far in excess of any likely to be found in apple juice!) may only cause transient inhibition of *Saccharomyces cerevisiae* while the organism is degrading the patulin.[17,73,182] It also has been shown that the degradation of patulin by yeast depends on protein synthesis and that the toxin is not degraded when added simultaneously with the protein synthesis inhibitor cycloheximide.[179]

Although it is probable that the degradation of patulin during a yeast fermentation in, for example, the manufacture of cider leads to loss of toxicity, it would still be useful to understand the nature of this degradation. Characterization of a degradation product could provide an indicator that cider was manufactured from poor quality apple juice in the same way that patulin itself is an indicator that a juice was manufactured from poor quality apples. Patulin reacts with free sulphydryl groups and with sulphur dioxide to give products that seem no longer to be toxic. Ascorbic acid also has been reported to reduce levels of patulin, but it is not yet clear what the breakdown products are. Although pasteurization (using high-temperature, short-time treatment of 10 seconds at 90° C) causes some reduction in patulin in fruit juices, it is only of the order of 20%, which is not sufficient to make a badly contaminated product acceptable.[72]

53.5 THE *FUSARIUM* TOXINS

Another important genus of mycotoxin producers is *Fusarium*, many species of which produce members of the trichothecene family of mold metabolites. Identification of species of *Fusarium* is not always straightforward, and several trichothecenes have been named after species of *Fusarium* now known not to produce them.[112] Thus nivalenol was thought to be produced by *F. nivale* (no longer considered to be a species of *Fusarium* but referred to *Monographella nivalis*!) and neosolaniol was thought to be produced by *F. solani*. Although fortunately not frequently found in foods, one of the most toxic of the *Fusarium* trichothecenes is T-2 toxin (Figure 53–2). This toxin is thought to have contributed to the epidemiology of the terrible outbreaks of ATA in Russia. In these instances, it was produced by species such as *Fusarium sporotrichioides* and *F. poae* growing on overwintered cereals. The trichothecenes are acutely cytotoxic and strongly immunosuppressive and, indeed, many of the deaths occurring during outbreaks of ATA were recorded as due to relatively trivial pulmonary infections of viruses and bacteria.

53.5.1 Deoxynivalenol

Deoxynivalenol (DON) (Figure 53–8) is a far more common, but much less toxic, trichothecene and is produced by species such as *F. graminearum* and *F. culmorum*, both of which also produce the estrogenic mycotoxin zearalenone (Figure 53–9). Although the LD_{50} of T-2 toxin is about 5 mg kg^{-1} in a wide range of animal species, that of DON is about 70 mg kg^{-1} in the mouse. An earlier name for DON was vomitoxin because it elicits a vomiting response in pigs at doses as low as 0.1 mg kg^{-1} bw and, indeed, is quite a potent feed refusal factor. Reports on the

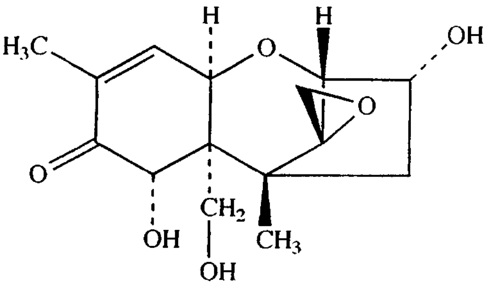

Figure 53–8 Deoxynivalenol

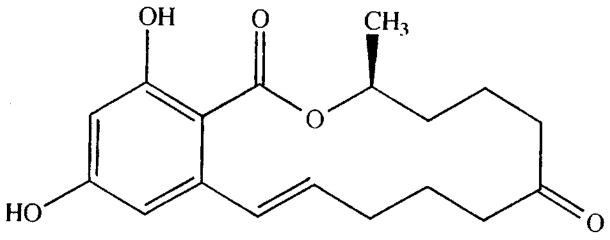

Figure 53–9 Zearalenone

occurrence of deoxynivalenol are shown in Table 53–10. The problem with assessing the significance of deoxynivalenol in foods is the frequent cocontamination with acetylated derivatives such as 3- and 15-acetyl-deoxynivalenol. The production of these two particular derivatives has been used to distinguish chemotypes of *F. graminearum* with interesting geographical distributions. Thus 3-acetyl-DON is associated with the Far East while 15-acetyl-DON is associated with strains from the US.[116] The first demonstration of 15-acetyl-DON in US corn implicated in feed refusal by pigs was considered to possibly "account in part for the refusal that could not be solely attributed to deoxynivalenol."[1]

The trichothecenes are remarkably stable compounds, and DON will survive both dry milling and wet milling processes of corn,[64] the experimental milling of wheat from which 76% is retained in the straight-grade flour.[160] The baking of bread has relatively little effect on trichothecenes such as DON.[51,85]

Table 53–10 Reports on the Occurrence of Deoxynivalenol and Related Toxins (ie, type B Trichothecenes)

Commodity	*Country*	*Levels*	*Reference*
Corn (moldy)	Italy	18 to 668 ppb	14
Corn (moldy)	US	mean 238 ppb	132
Barley	Korea	29 to 677 ppb	133
Barley	Japan	86 to 70,500 ppb	203
Wheat	USSR	100 to 1,390 ppb	187
Wheat	Japan	29 to 11,700 ppb	203
Wheat	Poland	9.6 to 25.3 ppm	138

53.5.2 Zearalenone

Zearalenone, which was first isolated as the agent responsible for vulvovaginitis in pigs, has very little acute toxicity, but there should be some concern about chronic exposure to a compound known to be estrogenic. It may be produced, together with DON and other trichothecenes, in a wide range of cereals including corn, barley, and wheat. A few examples of the occurrence of zearalenone are shown in Table 53–11. As well as estrogenic activity, zearalenone and its derivatives have anabolic, growth promoting activity. Both α-zearalanol (= zeranol) and α-zearalenol (marketed under the tradename Ralgro[170]) have increased anabolic activity compared with zearalenone itself and are used as animal feed additives in some parts of the world. Both these compounds are themselves natural products of species of *Fusarium*[12] and zearalenol is produced by the metabolism of zearalenone in the animal body.

53.5.3 Moniliformin

Moniliformin first was obtained from a strain of *Fusarium moniliforme* isolated from southern leaf blight-damaged corn seed as a water soluble toxin with an oral LD_{50} of 4.0 mg kg^{-1} in 1-day-old cockerels.[36] It also has potent phytotoxic activity. The remarkable structure (Figure 53–10) of this cyclobutane derivative was confirmed by single crystal x-ray crystallography and chemical synthesis of the potassium salt.[172] *F. moniliforme* is not a major producer of this toxin, but many other species of *Fusarium* produce it with, perhaps, *F. subglutinans*, *F. acuminatum*, and *F. avenaceum* being major producers.[30] Moniliformin can be quite a common contaminant of cereals and it was found in samples of Polish corn every year during a survey from 1985 to 1991 with levels ranging from 4.2 to 530 ppm.[101] A survey of 36 samples of retail corn products in the

Table 53–11 Reports on the Occurrence of Zearalenone

Commodity	*Country*	*Levels*	*Reference*
Corn (moldy)	Italy	156 to 7433 ppb	14
Corn (moldy)	USA	mean 904 ppb	132
Barley	Korea	40 to 1,416 ppb	133
Barley	Japan	105 to 15,300 ppb	203
Wheat	Japan	53 to 459 ppb	203
Wheat (moldy)	Poland	mean 0.29 ppm	139

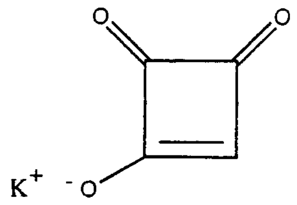

Figure 53–10 Moniliformin

UK during 1990 showed most samples to contain low but measurable levels of about 100 ppb with a maximum of 250 ppb in a single sample of cornmeal.[108] A further 55 samples of corn from Africa, Asia, and continental Europe also were analyzed during this survey. Although 30 of these samples contained no detectable moniliformin, a sample from the Gambia contained 3,160 ppb.

The LD_{50} for mice has been reported to be 20.9 mg kg^{-1} for females and 29.1 mg kg^{-1} for males. At toxic doses moniliformin causes rapid death without obvious overt cellular damage; although acute degenerative lesions in the myocardium are reported. This is particularly interesting because of the possible link between moniliformin and a human disorder known as Keshan disease seen in parts of China.[32] Members of the Liseola group of fusaria, including *F. moniliforme* and *F. subglutinans*, have been known to be very toxigenic despite not producing trichothecenes. They have been associated with esophageal carcinoma in humans, but the toxicology of pure moniliformin does not account for the observed biological effects of these organisms. The highly toxic and mutagenic metabolite fusarin C (Figure 53–11) seemed a good candidate,[65] but this also does not account for the carcinogenicity and other biological effects associated with culture filtrates of strains of *F. moniliforme*. The remarkable diversity of secondary metabolites of this group of fusaria was further demonstrated by the more recent isolation and characterization of the fumonisins.

53.5.4 Fumonisins

The most recently characterized mycotoxins of any major significance in human health are the fumonisins produced by species of *Fusarium*, such as *F. moniliforme*, which are known not to produce the trichothecenes.[60] Recent studies have shown that fumonisin production is variable within the different mating types of the perfect state of *F. moniliforme* (*Gibberella fujikuroi*)[99] and that it may be produced by quite a large number of species of *Fusarium*.[125] The genetic variability in the production of the fumonisins and fusarins also has been demonstrated with isolates of *F. moniliforme* from Indonesia, the Philippines, and Thailand.[117] Indeed, it has been shown to be produced by the unrelated mold *Alternaria alternata*.[31]

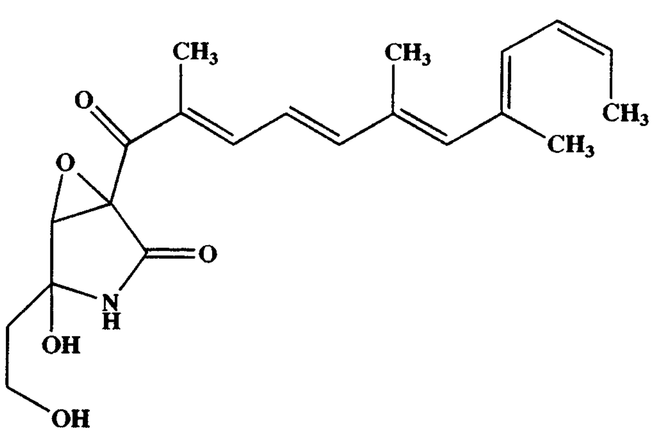

Figure 53–11 Fusarin C

Fumonisin B_1 (Figure 53–12) is water soluble, has considerable biological activity, is known to be responsible for equine encephalomalacia, porcine pulmonary edema syndrome, and hepatic cancer in rats, and may be involved in the epidemiology of esophageal carcinoma in humans in southern Africa[147] and parts of China, although, in the latter case, the correlation is not so convincing.[204] Now that methods for its analysis have become more widely available, it appears to be quite common in corn and corn products in Italy,[49] Portugal, Zambia, and Benin.[48] Indeed, Sydenham et al.[180] describe methods for the partial decontamination of bulk shipments of corn and also provide a useful bibliography of the occurrence and analysis of the fumonisins. Like a number of mycotoxins, the fumonisins are relatively heat stable and would not be significantly destroyed by drying processes for corn or heat treatments used for the production of maize derivatives.[50]

53.6 ANALYSIS

The diversity of chemical structure of mycotoxins, and the wide range of foods and animal feeds in which some of them may occur, usually need the development of different analytical methods for each mycotoxin in each food commodity. Interna-

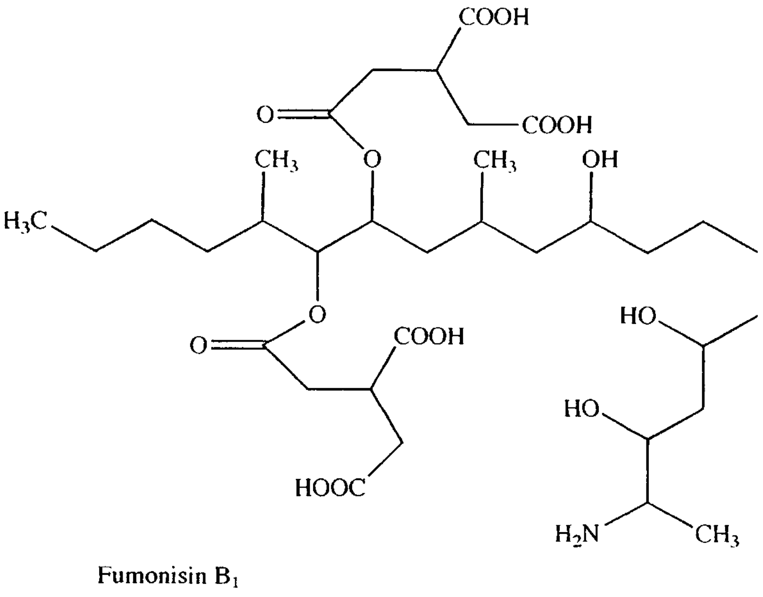

Figure 53–12 Fumonisin B_1

tional trade in many of the commodities most at risk from contamination requires the establishment of agreed methods of analysis, and many countries use those described in the Official Methods of Analysis of the Association of Official Analytical Chemists.[159]

53.6.1 Sampling

Before an analysis can be attempted, a sample must be obtained. The analyst frequently is presented with a single sample adequate in quantity for the analytical methodology, but without any guarantee that it is representative of the consignment from which it came. Many commodities to be analyzed are particulate in structure, and molds do not grow uniformly through such materials. Thus mycotoxins may not be distributed evenly throughout a consignment, although exceptions may be aflatoxins in milk and patulin in fruit juice. There is not even a normal distribution of mycotoxins within a batch of a commodity. For example, in the contamination of groundnuts, corn, cottonseed, or palm kernels with aflatoxin, it is likely that a relatively small number of kernels or seeds are contaminated with very high levels and many are not contaminated at all, giving a very skewed distribution.[82]

It is important to agree on a sampling plan that adequately protects the consumer without unnecessarily penalizing the producer. Satisfactory sampling plans, such as those devised for groundnuts by the US Department of Agriculture and the Tropical Products Institute (now the Natural Resources Institute) of the UK, define the size of the sample, how it is to be collected, a method for dividing the sample, and an analytical procedure. For many years sampling was not taken sufficiently seriously, but detailed research on the distribution of mycotoxins in commodities and an appropriate statistical analysis of the results have led to the increased use of acceptable sampling plans. The recognition of the extremely skewed distribution of mycotoxins has also led to the design of methods for spotting and eliminating the heavily contaminated kernels from consignments, thus ensuring that they do not contribute to the overall contamination of the batch after further treatment such as mixing, grinding, and oil extraction.

53.6.2 Extraction Procedures and Cleanup

Mycotoxins are produced in a complex matrix of macromolecules, including proteins, polysaccharides, and lipids, as well as being mixed with a range of low molecular-weight organic compounds of biological origin. Although many mycotoxins are readily soluble in water-immiscible organic solvents, it is not always easy to remove them from the macromolecular food matrix by direct extraction with simple solvents such as chloroform or ethyl acetate. It may be necessary to remove oils and fats by a preliminary extraction with hexane before extracting the mycotoxin. In the case of aflatoxin, detailed methods will vary from one commodity to another. Thus, defatted hazel nuts, pistachios, Brazil nuts, groundnuts, or groundnut meal can be extracted directly with chloroform after the addition of a small volume of water, whereas cottonseed and cottonseed products are best extracted initially with aqueous acetone and the aflatoxins subsequently partitioned into chloroform.

Those mycotoxins, such as ochratoxin A, that contain a carboxyl group can be extracted readily into chloroform after acidifying the commodity with aqueous phosphoric acid (0.1 M). Ochratoxin A can then be back-extracted from the chloroform using sodium bicarbonate solution or trapped on a column of diatomaceous earth impregnated with an aqueous solution of sodium bicarbonate. Esters and fat can be removed with hexane and chloroform and the acidic ochratoxin A eluted with a formic acid/chloroform mixture. It is worth noting that this method would miss ochratoxin C.

Some mycotoxins are sufficiently hydrophilic that they cannot be recovered efficiently with a simple chloroform extraction even though they are intrinsically soluble in chloroform. Among the trichothecenes, deoxynivalenol is best extracted initially with aqueous methanol, before subsequently partitioning into chloroform, and the fumonisins may be extracted from corn by soaking in distilled water for 2 to 3 hours with thorough mixing at intervals of about 30 minutes, or with methanol/water (80:20) for 30 minutes.

Cleanup procedures vary considerably depending on both the mycotoxin(s) and the commodity to be analyzed. A range of commercially available prepared columns, such as Sep-Pak, have proved very convenient as a cleanup stage for crude extracts of mycotoxins before subsequent analysis. The recovery of mycotoxins from all extraction and cleanup procedures needs to be validated; this may be done using spiked material prepared from clean samples of the commodity under study. It must, however, be recognized that it is usually easier to recover compounds from spiked samples than it is to recover the same mycotoxins from naturally contaminated material. For some mycotoxins, there are now samples available of naturally contaminated material, the mycotoxin content of which has been established by international collaborative studies.[62,63,190] The extraction and cleanup procedures usually will still provide a complex mixture requiring further separation before a specific physico-chemical method for the quantitative or qualitative analysis of individual mycotoxins can be applied. This further separation usually is carried out by some form of chromatography.

53.6.3 Chromatographic Methods

The most widely used technique is thin layer chromatography (TLC), in either one- or two-dimensional mode, but as they become more widely available, high-performance liquid chromatography (HPLC) and gas chromatography (GC) also have important roles in the analysis of mycotoxins.[35] A major advantage of HPLC is the possibility of automation, the use of autosamplers, and the possibilities of online cleanup of crude extracts and online derivatization before or after separation. There are well-established methods for the HPLC analysis of all the aflatoxins, zearalenone, the 8-keto trichothecenes, patulin (although resolution from 5-hydroxy methylfurfural can be a problem), and, most recently, for the fumonisins as their naphthalene 2,3-dicarboxyaldehyde derivatives.

For GC, the mycotoxins need to be sufficiently thermostable and volatile or be converted into volatile derivatives. The two most widely used detectors in mycotoxin analysis—flame ionization (FID) and electron capture (ECD)—do not require that a compound show any fluorescence or ultraviolet absorption, and the group of mycotoxins for which GC was initially the most suited were the trichothecenes, There is a large number of different trichothecenes, and ideally their analysis requires confirmation using mass spectroscopy coupled gas chromatography (GC-MS).[114] Capillary GC-MS offers such a sensitive tool now that nivalenol has been detected in samples of beer at concentrations as low as 0.1–0.84 ng ml^{-1}.[161] Recently the mass spectrometer has been applied as a sensitive confirmatory detector to HPLC for the detection of sterigmatocystin in cheese, bread, and corn products.[163]

53.6.4 Immunological Methods

Most of the traditional methods of mycotoxin analysis based on physico-chemical determination require the attention of a skilled analyst who would be hard pressed to complete the analysis of quite a small number of samples during a working day. The meaningful survey for the occurrence of mycotoxins in a commodity, or the routine monitoring of foods for the presence of mycotoxins, ideally requires the analysis of large numbers of samples and a number of biological assays,[148,149] and immunological approaches could make this possible.[33] Although mycotoxins are relatively small molecules, and hence not themselves immunologically active, they can form the haptens of an antigen by coupling to a suitable macromolecule such as bovine serum albumin. They may then present suitable epitopes to which specific antibodies can be raised. During the last few years, immunological methods have been developed for a wide range of mycotoxins including the aflatoxins, T-2 toxin, zearalenone, ochratoxin, cyclopiazonic acid, and, most recently, fumonisins B_1, B_2, and B_3.[6,52]

The specificity of monoclonal antibodies makes it possible to develop an analytical method for a single mycotoxin, such as aflatoxin B_1 in corn and groundnut meal or aflatoxin M_1 in milk and milk products. Even within chemically closely related structures such as the *Fusarium* trichothecenes, there is very little cross reactivity between a monoclonal raised to a single toxin such as T-2 toxin and other members of the family.[67] Both enzyme-linked immunosorbent assays (ELISA) and immuno-affinity columns are commercially available for a number of mycotoxins. ELISA is subject to interference from components in foods that may result in false positives,[94] but immunoaffinity columns are particularly useful as a very specific extraction, cleanup, and concentration stage in preparation for subsequent quantitative chromatographic analysis.

53.6.5 Safety Procedures

Mycotoxins should be handled as toxic substances and special care should be taken when handling pure toxins in a dry form or samples (including thin layer chromatography plates), which may be a source of dust. Accidental spillages of material containing aflatoxin generally can be treated with 1% sodium hypochlorite bleach, left for 10 minutes, swabbed down with 5% aqueous acetone initially, and subsequently rinsed with plenty of water.

Glassware exposed to aflatoxins can be rinsed with methanol, soaked in 1% NaOCl solution to which, after 2 hours, acetone is added to 5% of the total volume. The acetone is left for 30 minutes to destroy any potentially harmful but reactive intermediates formed by hypochlorite and then washed thoroughly. It is known, for example, that aflatoxin B_1 can form a carcinogenic derivative if treated with hypochlorite alone.

A detailed discussion of the laboratory decontamination and destruction of aflatoxins in laboratory wastes has been prepared for the IARC.[25]

53.7 SIGNIFICANCE AND LEGISLATION

The study of mycotoxins initially was concerned with isolated human illnesses, such as ATA, yellow rice disease, ergotism, and of the epidemiology of a number of illnesses in farm animals that could not be associated with such agents as infectious microorganisms, plant poisons, pesticide residues, or heavy metal poisoning. It gradually became apparent that molded animal feeds, or fungal infected forage in the field, could be responsible for illnesses such as sheep facial eczema, red clover disease, vulvovaginitis in pigs, feed refusal, turkey X disease, hepatocarcinoma in rainbow trout, and liver cancer in dogs. That these same compounds could be present in human foods, and possibly responsible for illness, only was appreciated when analytical methods made it possible to screen large numbers of foods and food components. There is no doubt that in tropical, developing countries, mycotoxins are responsible for acute illness and even the death of people although, even in these parts of the world, the numbers of people involved may be small compared with those who are ill and die from infectious diseases caused by protozoa, viruses, and bacteria.

In industrialized countries with a temperate climate, such as those of Northern Europe, it is difficult to assess the impact of mycotoxins on human health, although it is clear that they may have a political and economic impact. Thus the newspaper headlines "apple juice in cancer scare" was as much about the perception that there had been a coverup involving government as it was about knowledge of the occurrence and toxicity of patulin in apple juice. It was based on the finding that, of 32 samples of commercial apple juice analyzed, 5 had levels of patulin in excess of the advisory limit of 50 $\mu g\ kg^{-1}$ (they were 59, 82, 118, 153, and 434 $\mu g\ kg^{-1}$).[108] The presence of patulin in fresh apple juice implies the use of moldy fruit for its preparation; this is clearly undesirable. However, as we have already seen, the ecology of molds and their plant hosts, such as *Penicillium expansum* and apples, *Aspergillus flavus* and corn, and *Aspergillus parasiticus* and groundnuts, is more complex than postharvest molding of poorly stored commodities. These fungi may have a benign endophytic relationship with apparently healthy plants and low levels of mycotoxins may be produced as a result of stress, such as that caused by drought, of the crop during its development in the field. Because of this, there may be years when there is a detectable concentration of a mycotoxin in a crop even before it is harvested. Although there is no evidence at the moment that this is the case with patulin in apples, it has certainly happened with aflatoxins in corn and groundnuts. In 1980, the cost of aflatoxin contamination of corn in the South Eastern US arising in this manner was estimated at more than $237 million.[126] Because it would seem difficult to avoid contamination by aflatoxin in some commodities, attempts have been made to estimate the average daily intake for an adult in the US. The results suggest a level of 20 ng kg^{-1} bw for aflatoxin B_1, mainly from corn and corn products, and 0.8 ng kg^{-1} bw for aflatoxin M_1 from milk and milk products. It should be noted in this context that aflatoxin M_1 is less than one hundredth (1/100) times as carcinogenic as aflatoxin B_1.

Many countries have set legislative or recommended limits to the concentrations of a number of mycotoxins in foods (Table 53–12) and these may reflect analytical capability rather than a clear understanding of the toxicological significance or distribution of mycotoxins in foods. The inclusion of stachyobotriotoxin in animal feeds by Romania and phomopsin in lupin products by Australia reflect specific problems with these mycotoxins in those particular countries. Even for a single mycotoxin, such as aflatoxin, there is a wide range of maximum acceptable limits in different countries (Table 53–13). One of the major problems in setting legislative limits that will be acceptable to both the consumer and the producer is understanding the distribution of a mycotoxin in a bulk commodity, especially if it is particulate. Molds rarely grow uniformly throughout a commodity, and a mycotoxin will not have an even distribution. The study by Sharman et al.[165] showed that the batch average aflatoxin content for an 11-ton consignment of whole dried figs was 33 μg kg^{-1}, based on a single homogenized 20 kg sample made up of 20 × 1 kg subsamples. However, 67% of 200 boxes, each containing 12 kg and chosen at random from the 850 boxes making up the total consignment, had less than 10 μg kg^{-1}. Studies such as these demonstrate the need for accepted sampling plans to be incorporated into the analytical methodology and a number of such plans have been established.

Table 53–12 The Range of Regulatory Limits for Mycotoxins[144,189]

Mycotoxin	*Regulatory Limit (μg kg^{-1})*	*Number of Countries*
Aflatoxins in foods	0 to 50	53
Aflatoxin M_1 in milk	0 to 0.5	15
Deoxynivalenol in wheat	1000 to 4000	5
Ochratoxin A in foods	1 to 300	6
Patulin in apple juice	20 to 50	10
T-2 Toxin	100	2
Zearalenone	30 to 1000	4

Source: Data from A.E. Pohland, Mycotoxins in Review, *Food Additives and Contaminants*, Vol. 10, pp. 17–28, © 1993, and H.P. Van Egmond, Current Situation on Regulations for Mycotoxins, Overview of Tolerances and Status of Standard Methods of Sampling and Analysis, *Food Additives and Contaminants*, Vol. 6, pp. 139–188. © 1989.

At present, the UK limits for foods include a guideline level of 50 μg kg^{-1} for patulin in apple juice and a statutory limit of 4 μg kg^{-1} for aflatoxin B_1 in nuts, dried figs, and their products used for direct consumption. The latter is relaxed to 10 μg kg^{-1} for these commodities intended for further processing on the understanding that such processing will reduce the level to 4 μg kg^{-1} (Statutory Instrument [1992] No. 3236). These figures will be reduced to 2 and 5 respectively from January 1999. There is also a statutory limit for aflatoxin in animal feeds that varies depending on the animals concerned but is now set at 5 μg kg^{-1} when the feed is to be used for young animals and dairy cattle (Statutory Instrument [1991] No. 177).

A recent MAFF Food Surveillance Paper[108] describes and tabulates results from surveillance carried out from 1986 to 1992. The report shows that it is possible to find some samples of foods containing levels of a number of mycotoxins in excess of the guideline or statutory concentrations, but the numbers are small. Such findings demonstrate the need for continuing vigilance and effective surveillance coupled with open discussion of the significance of any results and an open mind to the implications of setting maximum tolerated levels. A detailed assessment of the occurrence and significance of mycotoxins to humans in the countries of the European Community has been prepared by Smith et al.[170] This report is a valuable source of information on a wider range of mycotoxins than could be included in this chapter and is supported by an extensive bibliography.

53.8 CONCLUDING REMARKS

Mycotoxins produced as a result of the molding of inadequately stored postharvest food commodities

Table 53–13 Maximum Acceptable Levels for Aflatoxin for a Selection of Countries*

Country	*Limit (μg kg⁻¹)*	*Notes*
United Kingdom	4*	Nuts, dried figs, and their products
	10*	As above but intended for further processing
United States	20	Total aflatoxins in all foods
	0.5	Aflatoxin M_1 in whole milk, lowfat milk, and skim milk
Australia	5	All foods except peanut products
	15	Peanut products
India	30	All foods
Japan	10	All foods
China	50	Rice, peanuts, corn, sorghum, beans, wheat, barley, oats

* By the end of 1997, it is probable that the European Community proposals to reduce these maximum admissible levels for aflatoxin B_1 to 2 and 5 μg kg^{-1}, respectively, and to set a limit of 0.05 μg kg^{-1} for aflatoxin M_1 in milk, will come into force (DOC. VI/1669/97-rev. 4). These levels were agreed on in July 1998 and came into force in January 1999.

can reach concentrations that may have acute and chronic significance in the health of humans. This situation is most likely to occur in the tropical humid climates and will pose a problem for the poorer peoples of the world in developing countries. Mycotoxins also may be produced in the field before harvest at concentrations that may not be toxicologically significant; this phenomenon may be difficult to control. However, the ability to quantitatively detect very low levels, and concern over long-term effects such as carcinogenicity, genotoxicity, and immunosuppressive activity, have led to stringent advisory and legislative levels in a number of countries. These will have an effect on world trade and, although they may protect people in the wealthier parts of the world from exposure to mycotoxins, they may increase the problems for the developing countries, at least in the short term.

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54 Fish and Shellfish Poisoning

John Liston

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54.1 INTRODUCTION

Fish and shellfish poisoning are worldwide problems. Indeed, it appears that there is no part of the world ocean that does not experience periodic incursions of toxicity, which result in edible species of fish or, more commonly, shellfish becoming dangerous for consumption by humans and usually requiring government action to close, or greatly restrict, harvesting and distribution of affected shellfish or fish. Unfortunately, the incidence of toxic contamination seems to be on the rise because toxigenic microalgae, which are the primary source of the toxins, spread into previously unaffected areas.[36,115,128]

The foodborne illness discussed in this chapter is caused by consumption of fish or shellfish containing natural toxins derived from microorganisms and accumulated in the living animal, in most cases, through the food chain. The initiating factor usually is rapid multiplication of the toxigenic organisms, resulting in a bloom. The occurrence of the toxins is not consistent in any particular species of fish or shellfish but is a consequence of exposure of individual animals to toxin-producing microorganisms or toxin-carrying animals used as food. In general, the presence of toxin in fish or shellfish is not evidenced by any easily observed abnormalities in behavior or appearance of the living animal or of the separated tissues. Moreover, the raw or cooked flesh and other tissues appear normal and cannot be distinguished organoleptically from those of nontoxic animals. The toxins are relatively small organic molecules with a high potency for intoxication of humans but of little or no immunogenic capacity. There is no evidence of acquired immunity in any patient recovering from fish or shellfish poisoning and repeated episodes are common. The toxins typically are stable compounds unaffected by cooking or processing that pass easily into the internal tissues. Most are principally neurotoxins, but they often also give rise to gastrointestinal symptoms. Not infrequently, symptoms are due to mixtures of different toxic substances present in the animal tissues consumed by the patient. The toxins in such mixtures are usually chemically related substances but occasionally are quite different. This can give rise to variation in the reported frequency and types of symptoms.

Fish and shellfish poisonings are thus characterized by uncertainties and uncontrollable factors that are not encountered in most other microbiologically caused food poisonings. With one exception—scombroid poisoning—toxin accumulation occurs in the ocean and involves toxigenic microorganisms, usually microalgae, that are outside human control.

The principal intoxications resulting from seafoods having a microbiological origin include paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP) (also known as domoic acid poisoning), ciguatera, scombroid (histamine) fish poisoning, and puffer fish poisoning. PSP, DSP, and NSP are caused by toxins produced by dinoflagellates and ASP by a diatom toxin. These diseases typically result from consumption of bivalve mollusks that have been feeding on the toxigenic algae. Ciguatera is caused by eating fish from tropical waters that have accumulated toxins through a food chain. The toxin(s) causing ciguatera are derived from toxigenic microalgae growing in and around tropical coral reefs and are passed up the marine food chain through herbivorous reef fish to more far-ranging carnivorous species. Histamine or scombroid poisoning is caused by consumption of fish containing high levels of histamine (and other biogenic amines) resulting from histidine dehydrogenase activity of bacteria multiplying on the fish after death. Puffer fish (fugu) poisoning results from a toxin, tetrodotoxin, which is produced by bacteria probably growing in the intestine of the living fish.[113,127] A summary of the general characteristics of these poisonings is provided in Table 54–1.

54.2 SHELLFISH POISONING

The identification of microalgae capable of producing the various toxins and the toxins themselves implicated in human disease outbreaks from shellfish is a continuing process because the appearance or recognition of new toxic diseases and an increasing scientific investigation of both the microorganisms and the toxins is ongoing. There is some evidence that toxigenic microalgae are spreading into different coastal areas and the incidence of toxic blooms is increasing on a worldwide basis.[2] The causes of these phenomena may include the warming of waters in the Pacific because of the recurrent El Niño phenomenon and human effects such as translocation of species by ballast water and eutrophication from pollution.[2,4,37,109] The practical importance of all this for the working microbiologist is the necessity of keeping an open mind concerning the linkage of outbreaks to blooms of particular algae or other microorganisms in determining cause and in choosing a course of action.

Dinoflagellates are responsible for most shellfish poisoning. They are single-celled photosynthetic organisms that are approximately spherical, about 50 μm in diameter, and frequently are encased (armored) in a theca made up of connected plates. There are characteristically two grooves in the surface, one vertical and the other horizontal, around the middle of the plant, the girdle. The microorganisms are made motile by two flagella. There is a pore toward a pole of the cell, the apical pore. Dinoflagellates are designated as species largely on the morphological data of arrangement of thecal plates, groove shapes and alignment, and the appearance of the pore structure. Dinoflagellates are part of the phytoplankton that live and grow most commonly in the upper layer of the ocean, but some are benthic (bottom dwellers in shallow waters). They lie at the base of the food chain and are consumed by zooplankton such as copepods and by herbivorous fish, directly or indirectly. They are consumed by filter-feeding shellfish such as mussels, clams, oysters, and scallops near shore in shallow waters. Plankton-feeding fish may accumulate toxic dinoflagellates in the stomach and intestine during feeding.

54.2.1 Paralytic Shellfish Poisoning

Organisms and Their Toxins. PSP is not only the most widespread geographically of the marine toxications, but also is probably longest recognized and most widely studied. Cases due to consumption of mussels were reported in France as early as 1689 and later (1790–1903) in northwest America, the British Isles, Scandinavia, Japan, and South Africa.[39] However, the connection between the disease and toxigenic dinoflagellates was not recognized until the 1920s when the work of Meyer and Sommer[74,119] resulted in a specific test for the toxin and opened the field of research. Initially, it was thought that only dinoflagellates of the genus *Gonyaulax* were responsible for PSP;[102] however, *Prorocentrum micans* was implicated in a PSP outbreak in Portugal in the 1950s, and subsequently other genera and species of microalgae were shown to produce PSP toxins.[109]

The armored dinoflagellates *Gonyaulax* spp. (*Protogonyaulax*), now renamed *Alexandrium*, are probably the dominant cause of PSP outbreaks in temperate water regions. *Alexandrium catenella* (first identified as a PSP cause in 1937[119]) and *A. tamarense* remain the most frequently implicated species for PSP outbreaks in North America (Pacific and Atlantic coasts, respectively) and Japan and, to a lesser extent, Europe, South America, and elsewhere.[84,109] *A. minutum*, reported widespread distribution includes Egypt, North America, France, Spain and Portugal, Italy, Turkey, Ireland, the Netherlands, Australia and New Zealand, and the Baltic Sea.[16,27,38,109] Other *Alexandrium* species have been identified as toxigenic mostly in Europe but are less frequently encountered in PSP incidents involving human disease.

Gymnodinium catenatum is another PSP-causing dinoflagellate. First detected off Mexico in 1940, it was associated with repeated PSP outbreaks in Spain during the 1980s and subsequently with toxic outbreaks in Mexico, Japan, Portugal, and Tasmania.[3,35,38,73]

Pyrodinium bahamense v. *compressum* has been implicated in numerous PSP outbreaks, mainly from tropical areas. The organism originally was isolated in the Bahamas and was first identified as a cause of illness and deaths in outbreaks in Papua New Guinea.[67] Subsequently, it has been associated with recurrent outbreaks throughout the Indo-West Pacific region in the Philippines, Indonesia, Malaysia, Fiji, Guam, and other island communities in the area.[25,67] The outbreaks have been characterized by significant mortality and the unusual involvement of free-swimming fish as a source of the human intoxication. This is due to the regional practice of consuming pelagic, plankton-feeding fish whole with intestines included. There was a severe outbreak of PSP involving deaths in Guatemala in 1987.[100] This organism continues to present a problem in tropical areas among people who are greatly dependent on fish and shellfish for subsistence and as a livelihood.

Although not yet implicated in human PSP outbreaks, two fresh water cyanobacteria *Aphanizomenon flos-aquae* and *Anabaena circinalis* have been shown to produce saxitoxins.[6,44] *Anabaena circinalis*, when fed experimentally to an edible fresh water mussel, has been shown to cause accumulation of saxitoxins in the mussel tissues at levels toxic for humans and a similar accumulation was earlier demonstrated in *Mytilus edulis* fed *A. flos-aquae*.[83,105]

The possibility that bacteria may be involved in the production of PSP toxins has been raised repeatedly.[56] It has been reported that toxigenic algae in culture produce more toxin when bacteria are present than when grown axenically, and there have been claims that intracellular bacteria are instrumental in algal toxin production.[55,110] The evidence for this latter claim is weak, but bacteria isolated from unialgal cultures (sometimes supposed axenic) have been shown to produce PSP toxins. A strain identified as *Moraxella* sp. produced saxitoxin, which was detected by analysis of the growth medium by electrophoresis, thin-layer chromatography, high performance liquid chromatography (HPLC), and mouse bioassay.[55] Similarly, a *Bacillus* strain also produced PSP toxins in culture.[55,86] The evidence that *Alexandrium* spp. can produce toxin axenically seems well established, and at least one study refutes the claim that more toxin is produced when bacteria are present.[114] Moreover, recent genetic studies of toxin production support a hereditary basis for this in *Alexandrium*.[47] Nevertheless, the involvement of bacteria cannot be completely ruled out.[127]

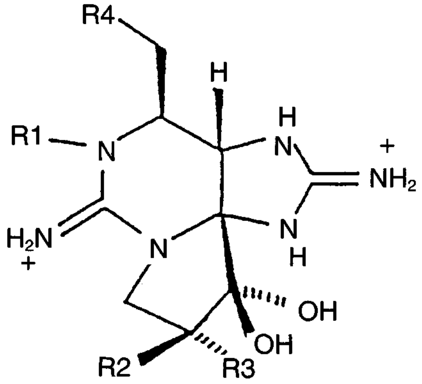
Saxitoxin, isolated from toxic Alaskan butter clams (*Saxidomus giganticus*), was long thought to be the major cause of PSP.[106] It is now known that the disease involves a family of toxins that may be considered derivatives of saxitoxin (Figure 54–1). There is an excellent discussion of the chemistry and pharmacology of this toxin group in the literature.[35,89] Toxigenic algae of the *Alexandrium* group show characteristic toxin profiles depending on the strain; this is probably genetically determined[14,15] and controlled by nuclear DNA.[47] The principal toxins found in algal strains implicated in human disease include saxitoxin, neosaxitoxin, and gonyautoxins 1 through 4, and these are most potent when tested by mouse bioassay. The B and C sulfocarbamoyl toxins are less toxic by an order of magnitude while the decarbomoyl derivatives are about half as toxic as the parent toxins.[11,115] This ranking is believed to correspond to the human response also.

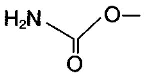
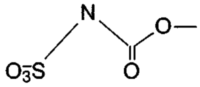
All saxitoxins act by binding to the surface of voltage-activated sodium channels in nerve cells and thereby interrupt the flow of Na^+ ions, which stop signal transmission.[35,106] The details of attachment and the mechanism of blocking are still not fully understood. Attachment is apparently reversible since, except where death ensues, symptoms disappear within hours or days and no further toxic effect is observed.

The route of transmission of toxin to human consumers is quite direct.[35] The first event is the development of a toxic algae bloom resulting from rapid multiplication to populations of 10^3 or greater cells l^{-1} of seawater in response to suitable conditions. Unfortunately the nature of suitable conditions has not been determined except that in temperate regions' water temperatures above 14° C, and calm weather in spring, summer, and autumn usually are required. Blooms may be associated with discoloration of the water because of the large number of microrganisms formed. This often is referred to as red tide, but the color may range from red to brown or greenish shades. Red tides do not necessarily presage a toxic condition because a number of nontoxic microalgae and cyanobacteria can be involved, but they do indicate the occurrence of water conditions suitable for rapid microalgal growth. Most of the dinoflagellates causing PSP produce resting cyst

Table 54–1 Summary of Types of Fish and Shellfish Poisoning

Disease	*Food Vehicle*	*Agent Producing Toxin*	*Chemical Nature of Toxin*	*Symptoms*	*Occurrence*
Paralytic shellfish poisoning	Bivalve mollusks	Microalgae and bacteria in living shellfish	Saxitoxin, neosaxitin, gonyautoxins	Neurologic paresthesia, paralysis death by asphyxiation	Worldwide
Diarrhetic shellfish poisoning	Bivalve mollusks	Microalgae in living shellfish	Okadaic acid, dinophysis-toxin	Gastrointestinal diarrhea, nonlethal	Europe and Japan
Neurotoxic shellfish poisoning	Bivalve mollusks	Microalgae in living shellfish	Brevetoxins	Neurologic hot-cold reversal, paresthesia	US, New Zealand
Amnesic shellfish poisoning	Bivalve mollusks	Diatoms in living shellfish	Domoic acid, isodomoic acids	Neurological memory loss, gastroenteritis death by brain-stem damage	Canada, Western US, New Zealand
Ciguatera fish poisoning	Tropical reef fish and predators	Microalgae in reef vegetation	Ciguatoxins	Neurological hot-cold reversal, complex symptoms, not usually lethal	Tropical Pacific, Caribbean
Scombroid fish poisoning	Active (pelagic) free-swimming fish	Bacteria growing on fish after death	Histamine	Allergy like, and enteric symptoms, nonlethal	Worldwide
Puffer fish (Fugu) poisoning	Puffer fish	Bacteria associated with living fish	Tetrodotoxin	Neurological, anhydro-paralysis, paresthesia, death by asphyxiation	Japan, SE Asia, elsewhere



R1	R2	R3	Carbamate Toxins	N-Sulfocarbamoyl Toxins	Decarbamoyl Toxins
H	H	H	STX	B1	dc-STX
OH	H	H	NEO	B2	dc-NEO
OH	H	OSO_3^-	GTX I	C3	dc-GTX I
H	H	OSO_3^-	GTX II	C1	dc-GTX II
H	OSO_3^-	H	GTX III	C2	dc-GTX III
OH	OSO_3^-	H	GTX IV	C4	dc-GTX IV
			R4: 	R4: 	R4: HO—

STX = saxitoxin, NEO = neosaxitoxin, GTX = gonyautoxin, dc = decarbamoyl

Figure 54–1 Paralytic Shellfish Poisoning Toxins. *Source:* Copyright © 1992, M. Beitler and J. Liston.

forms that persist in upper sediments or are suspended in water, creating seed beds that germinate to form active reproducing forms when conditions are appropriate. A phenomenon encountered increasingly in some temperate regions (particularly North America) is winter or cold-weather shellfish toxicity; this is believed to be due to mollusks feeding on cysts perhaps stirred from the sediment by storm surges. Cysts do contain toxin. In most cases molluskan shellfish ingest dinoflagellates or their spores from ambient water in their normal feeding mode, which is essentially passive, involving the filtering out of suitably sized particles. The ingested food passes more or less slowly through the alimentary and digestive system, and some toxin accumulates in the digestive glands or passes into internal tissues, though much passes directly out in feces. Most mollusks show a transient toxicity, which is lost within days, or at most weeks, after the bloom subsides. Some mollusks retain toxicity for longer periods; in particular, *Saxidomus* spp (eg, butter clams) store saxitoxin mostly in the siphon and may remain toxic for several months or, in extreme cases, for over a year.[12] Toxin passes to the human consumer directly from the ingested mollusk. Typically mollusks are consumed whole either raw or after cooking, which unfortunately does not affect the toxin. Illness ensues if enough toxin is ingested and the severity of the disease depends on the total consumed. Nevertheless, this is a very dangerous toxin and death in adult humans can follow ingestion of only 2 mg; individual shellfish have been reported to contain in excess of 10 mg.[84]

Characteristics of the Disease. This disease occurs most commonly in individuals who have eaten toxic mollusks, including mussels, clams, oysters, and scallops. In some cases in the Philippines and adjacent regions, the disease has resulted from consumption of whole plankton-feeding fish, including viscera. Since the PSP toxins are heat-resistant, both cooked and uncooked seafood can cause the disease. Symptoms appear between 5 and 30 minutes after ingestion of toxic mollusks (or fish), and their severity and prognosis depend on the amount of toxin consumed. Saxitoxins are among the most potent human poisons but it is difficult to determine doses necessary for symptoms to appear. Moreover, there is variability in the response of individuals, influenced by whether consumption was on an empty (more sensitive) or full stomach and whether oil (good) or alcohol were consumed. Another complicating factor is the variability in the actual mix of toxins in the consumed foodstuff. As has been noted earlier, there is strain specificity in the toxin profile of toxigenic dinoflagellates, and the pattern of toxin occurrence in different mollusk species feeding on the same dinoflagellate strain may also vary.[12,15,50,125] It appears from the literature that as little as 120 to 180 μg saxotoxin have caused moderate symptoms and 0.5–2 mg have caused death. Most regulatory authorities require that shellfish harvest cease when toxicity in mollusks rises to 80 μg STX 100 g^{-1} edible tissue.

In mild cases of PSP, patients experience a tingling or burning sensation of the lips, gums, and tongue that progresses to the neck, arms, fingertips, and toes and changes to numbness later. Occasionally, headache and dizziness are reported. In moderate cases, numbness extends to the arms and legs, making voluntary movements difficult; ataxia is accompanied by a peculiar feeling of lightness (floating on air). Aphasia, muscle pain, salivation, headache, thirst, nausea, and vomiting may occur. Severe poisoning causes progressive paralysis, ultimately leading to respiratory difficulty. Victims are conscious and aware throughout the illness. In untreated cases, paralysis may become progressively more severe, and death occurs due to respiratory paralysis, usually within 12 to 18 hours. If the patient survives beyond 12 hours, the prognosis is good and recovery generally is uneventful without sequelae.[39]

Treatment is mainly symptom directed and supportive. The stomach should be emptied by gastric lavage, and the patient placed in a respirator. Artificial respiration at the onset of symptoms is essential in severe cases and can prevent death even when very high doses have been ingested.[129] Toxin is apparently released from the site of attachment on Na+ channels and excreted without damage to nerves or tissues. Patients normally recover within hours or, at most, a few days. Nevertheless, it should be noted that saxitoxins are powerful and dangerous. Although death rates are low, perhaps less than 2%, in areas where shellfish regularly become toxic and the population is well aware of the risks and how to avoid them, rates as high as 23% have been reported in outbreaks occurring among uninformed populations. Statistics of global incidence of PSP are not readily available, but outbreaks are reported each year from different countries involving from a few to more than a hundred cases. The usual pattern is that an outbreak, often with deaths, alerts both local shellfish harvesters and regulatory authorities to the hazard and either warnings or an actual control system is put in place. Subsequently, there continue to be reports of toxic shellfish periodically in the region but disease incidence drops and deaths are rare. Where a mollusk culture industry is in place, control can be particularly effective because it is not in the best interest of growers that illness be associated with their products, and incidents of PSP poisoning almost exclusively involve recreational or subsistence gatherers.[84]

Food Vehicles and Transmission to the Consumer. The four shellfish poisonings—PSP, NSP, DSP, and ASP—share common features that determine the food vehicles and transmission routes to the consumer. Disease usually is due to the consumption of toxin-containing bivalve mollusks, such as mussels, clams, and oysters, all of which are sessile animals growing and feeding in fixed positions in or on the seafloor, on rocks, or attached to strings, rafts, or other containers in aquaculture operations. Some incidents have been due to slightly more mobile invertebrates such as scallops and snails; more rarely, crabs have also been implicated; however, these animals are quite limited in their feeding range. In many cases, the blooms of toxic algae are quite circumscribed, occurring in bays or other areas of confined waters where conditions are suitable for rapid and concentrated growth because of calm and undisturbed water. Outbreaks are thus often assignable to limited areas of origin where toxic mollusks are found. Moreover, in temperate regions, there is strong seasonality in the pattern of occurrence with most outbreaks occurring in summer and autumn when coastal water temperatures are elevated; this is true except in the case of ASP where the causal diatoms seem to bloom preferentially in colder waters. Microalgal blooms tend to be limited in duration corresponding to the persistence or otherwise of the water conditions supporting growth.

The risk to consumers of disease is thus confined, in the main, to shellfish eaters ingesting animals from toxic beds during periods of blooms. Once an area has been identified as toxic, the risk effectively disappears because harvest is then prohibited. Unfortunately, because of the stability of marine toxins, the risk from eating toxic mollusks cannot be reduced by cooking or otherwise processing the seafood. There is some reduction in risk by not consuming the viscera because a large proportion of toxin is usually in the stomach, intestine, and related internal organs, but this will not eliminate all danger because toxin may accumulate in clam siphons and other tissues. Mussels, clams, and oysters typically are eaten whole and are probably the most common vehicles of shellfish intoxications. In North America and, increasingly, elsewhere, only the adductor muscle of scallops are eaten and intoxications due to this mollusk are rare; however, in some countries the whole animal is eaten and the risk is correspondingly higher.

Saxitoxins have been identified in 110 species of sessile mollusks, ascidians, and barnacles; 27 species of decapods; 16 gastropods; 19 finfish; 1 cephalopod; and 1 starfish.[11] Fifty-four of these species have been involved in human disease; 34 in deaths. The frequency of involvement in human illness was calculated to be: clams, 35%; scallops, 24%; mussels, 21%; oysters, 12%; and cockles and others, <6%. The finfish species implicated in PSP all were involved in *Pyrodinium*-caused outbreaks in the Philippines, Indonesia, and Malaysia. PSP originally was known as mussel poisoning because these mollusks principally were involved in outbreaks recorded early in Europe and North America. They accumulate toxin more rapidly and to a higher level than other mollusks and they are tested as indicator animals for PSP in a number of countries. Indeed, mussel collection is prohibited during the summer months in California when the danger of toxic algal blooms is high.[84] Mussels also clear themselves of toxin rapidly when blooms subside, and toxicity in clams can persist longer, representing a potential danger if mussel results are relied on as a basis for closing and opening harvesting.

Detection and Control. The control of shellfish poisonings depends on surveillance of both waters and animals. This is possible because shellfish are localized along shorelines that are accessible to inspection and specimen gathering and because the presence of high numbers of causative, toxigenic microalgae becomes evident as a bloom in the inshore waters. Surveillance may involve collection of plankton samples, which are then examined for the presence of potentially toxic microalgae. If these are found, they are enumerated and, if sufficiently numerous, plankton samples may be tested directly for toxicity. Identification of toxic algae is mainly by direct microscopic examination of plankton samples and this requires expertise that may not be available in many small countries. Fortunately, the Intergovernmental Oceanographic Commission of UNESCO sponsors a number of training courses for field sampling and identification of harmful algae specifically for developing countries. Nevertheless, the identification of toxic microalgae can be difficult even for experts, and more objective methods that are less dependent on visual observation of whole cells are desirable. Methods based on isoenzyme electrophoretic patterns, toxin composition, and cell surface antigens all have been employed with variable effectiveness. Lectins have been applied effectively for typing bacteria and protists, and recently Costas and Rodas[19] have reported good results using fluorescent lectins for the identification of toxic dinoflagellates. Newer techniques for toxin analysis with very low detection limits facilitate the direct analysis of plankton samples.[9,92,117]

Shellfish may be sampled using a number of different protocols depending on the particular toxin(s) expected and such factors as season of the year, likely distribution of harvested shellfish, whether aquaculture or other commercial production is involved, and the extent of direct recreational or subsistence harvesting. Past experience of shellfishborne disease often will influence where and how frequently sampling and toxin testing are undertaken; however, recent experiences of sudden and unexpected incidence of novel toxicities in a number of countries in Europe and Australasia caused regulatory agencies to institute multiple toxin testing on a routine basis.[21,66] Toxin testing may be limited to so-called indicator species, most commonly mussels, which are generally the first to become toxic when a bloom occurs. Where commercial production of other species such as oysters is important, these species will normally be tested routinely (eg, the US). This is particularly important where cultured or commercially harvested shellfish are distributed to markets across state or international boundaries and cannot be recalled rapidly or embargoed if toxin is found. Where there is a high risk of toxicity, commercial producers may be required to submit samples for analysis before shipment. In some cases mussels are not suitable indicators (eg, for domoic acid in Washington state shellfish), and a broader range of shellfish should be tested. A determination that it is safe to resume harvest of shellfish for human consumption must depend on analysis of the actual species to be harvested.

The most widely used method of analysis is by animal testing, usually involving mice.[5] This has the advantage, from a public health standpoint, of measuring actual toxicity without regard to toxin composition, and, assuming that the effect on mice is equivalent to that on humans, it is the best test of risk. The symptoms shown by the mouse and the time to death can, when considered together with the source of the toxin, establish the toxin's identity. The results may be quantitated through dilution of toxin extracts and expressed as mouse units (MU) or as a chemical concentration when toxins have been purified. Extracts are prepared using appropriate methods for the expected toxins: aqueous for water soluble and organic solvents for others.

Methods for the extraction and testing of PSP toxins are well established and described in standard manuals such as the Association of Analytical Chemists (AOAC).[5] The extract is prepared by homogenizing shucked and washed shellfish meats in 0.1N HCl, boiling to precipitate proteins, diluting the cooled preparation, and centrifuging to clear precipitate. After appropriate dilutions calculated to achieve a death time of 5 to 7 minutes, 1 ml of the supernatant is injected intraperitoneally into the mice. Actual time of death is measured and converted into MU from a standard table. The assay requires a saxitoxin (STX) standard, which is available commercially. The MU for PSP is defined as the amount of toxin required to kill a 20 g mouse in 15 minutes. Mouse units may be converted to a quantitative measure defined as μg STX equivalent 100 g^{-1} tissue by multiplying by a correction factor, CF, which is 0.16 μg for the Webster strain of mice used in North America. Different values may be necessary for other strains (eg, 0.34 for the ddY strain used in Japan).[82] The method is wasteful of mice because of the need for testing multiple dilutions and there are other biological factors that contribute to the high average variability of ± 20%. Nevertheless this is still the officially accepted assay for determining mandatory closure of harvesting of edible shellfish in most countries, including the US.

Other animal assays for PSP toxins have been tested using house fly, cockroach, brine shrimp, finfish, and domestic chicken but none has proved sufficiently sensitive or practical for general use. Young chicks appear to have equal or greater sensitivity to PSP toxins as mice and have been used in emergency situations to test shellfish safety.[34] A number of assays that depend on measurement of the sodium channel-blocking effect of PSP toxins have been proposed, and most recently interest has focused on the method proposed by Kogure et al.[57] In this method, cultured mouse neuroblastoma cells are treated with the sodium channel activator veratridine in the presence of ouabain, an inhibitor of the Na^+/K^+ adenosine triphosphatase (ATPase). The effect is an elevation of intracellular sodium levels, causing swelling and eventual death of the cells. STX, by blocking Na^+ flow, protects against this effect in a dose-dependent manner, permitting quantitation. The original test required visual scoring of 200 or more cells per sample, which was quite tedious because of cell morphology variability. Two research groups independently developed colorimetric techniques to facilitate the use of dyes, crystal violet,[49] and neutral red[29] to stain the cells, permitting the use of microplates and automated reading of results. Most recently, a method making use of the reduction of a tetrazolium compound (3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium, Sigma) to a blue-colored formazan by actively metabolizing cells has been described and tested extensively.[69,70] This method also was reported independently by another research group.[138] The method is slower than the mouse bioassay requiring 24 to 48 hours but is animal-friendly, more sensitive, and convenient. This method also can be used for tetrodotoxin and Na^+ flow activators such as brevetoxins and ciguatoxins.

Perhaps the most widely used procedure for analysis of PSP toxins is HPLC. The method depends on a postcolumn derivatization of separated toxins to fluorescent compounds by oxidation under alkaline conditions. Current procedures are based on that described by Sullivan and coworkers;[124,126] the most recent method with widest recognition of STX and derivatives is that of Oshima.[89] The HPLC procedure provides for identification and quantitation of individual toxins and is particular useful for investigative and research purposes. Numerous immunoassay procedures for STX have been published, and commercial kits based on enzyme-linked immunosorbent assay (ELISA) methodologies are available in Europe and Canada; however, none has yet achieved general acceptance. This is due to difficulties in dealing with the various toxin mixtures produced by different strains of toxigenic algae and resulting from metabolically induced changes of toxins in shellfish tissues.[15,124]

54.2.2 Diarrhetic Shellfish Poisoning

Organisms and Their Toxins. DSP is perhaps the second most widely reported shellfish intoxication, but was only recognized in the 1970s from outbreaks in Japan and soon thereafter in Europe.[51,139] The disease is caused by ingestion of molluskan shellfish contaminated by toxins from the dinoflagellates *Dinophysis* spp. and *Prorocentrum lima*. Blooms occur in summer and autumn, and it has been reported that as few as 200 microalgae l^{-1} of sea water are sufficient to create toxic conditions in shellfish.[1] The duration of toxic blooms and persistence of toxins is longer than for most PSP incidents but the symptoms, which resemble those of gastroenteritis, are less severe.

The number of *Dinophysis* species implicated as sources of toxin has continued to increase as the DSP incidents have been reported in different parts of the world. *D. fortii* was the first implicated but *D. acuminata*, *D. acuta*, *D. mitra*, *D. norvegica*, *D. rotundata*, *D. sacculus*, and *D. tripos* all have been shown to be toxic.

Prorocentrum lima was associated early with incidents of DSP[81] and seems to be particularly prominent in mussel culture areas. It has a wide distribution being reported from Europe, North and South America, Japan, and New Zealand.[109]

The toxins causing the diarrhetic symptoms characteristic of DSP are polyethers, particularly okadaic acid, and dinophysistoxins shown in Figure 54–2. Other toxins have been identified in toxic *Dinophysis* spp. and in *P. lima*, including pectenotoxins and yessotoxins, all of which are also polyethers and are extracted by lipid solvents.[137] The chemistry and properties of the toxins are succinctly presented by Yasumoto et al.[138] Most commonly, okadaic acid (OA) has been reported as the primary cause of diarrhea in Europe and dinophysistoxin (usually I) in Japan, but both occur together. Pectenotoxins are reported to be hepatotoxic, and yessotoxins are highly toxic in mice, but neither group has been shown definitively to be active in human DSP.[137–139] The toxins show similarities to other polyether marine toxins specifically brevetoxins (NSP) and ciguatoxins (ciguatera). The profile of DSP toxins is somewhat variable among different strains, although OA seems to be present consistently. Quite low populations of dinoflagellates can cause shellfish to become toxic.

Characteristics of the Disease. This disease has been reported primarily from toxic mussels, but other bivalve mollusks including oysters and clams and scallops also have been implicated. The symptoms are similar to those due to gastroenteritis of bacterial origin, and this probably delayed identification of the syndrome until the 1970s. Patients have been reported to experience diarrhea (92%), nausea (80%), vomiting (79%), abdominal pain (53%), and chills (10%).[139] The symptoms appear 30 minutes to 12 hours after consumption of toxic shellfish, in most cases (70%) within 4 hours. Symptoms disappear within 3 days and there are no sequelae. This is thus a mild disease essentially without mortalities; however, because the two toxins principally involved—OA and dinophysistoxin 1—have been demonstrated to have potent tumor-promoting activity, there is concern that chronic exposure may induce tumor formation in the digestive system.[123] Pectenotoxins are highly hepatoxic and yessotoxin is highly toxic to mice, raising concerns about these DSP-associated substances although no human effects have been demonstrated.[138]

Toxigenic dinoflagellates producing DSP toxins are widespread through temperate waters of the world, but outbreaks of human disease due to DSP have been limited mainly to Japan and Europe. In Europe, DSP has been a particular problem for the mussel-growing industry in Spain and France. Several thousand cases occurred each year through the 1980s but the imposition of shellfish harvesting controls in the affected countries in recent years has greatly reduced incidence of DSP.[21,109]

Food Vehicles and Transmission to the Consumer. Diarrhetic toxins have been identified dominantly in mussels but also in oysters, scallops, and clams. Retention of toxins in mussels was reported to be very short—perhaps one week;[109] however, toxicity of mussels can persist possibly for a month or longer if sufficient populations of *Dinophysis*, which may be as few as 200 *Dinophysis* cells l^{-1}, are in the water.[1] Moreover depuration studies carried out in Italy indicated a requirement for >30 days in a pond system and >5 days when an ozonization technique was used to ensure elimination of the toxin.[20]

Detection and Control. In most countries where DSP has occurred, particularly in Europe (eg, France, Spain, Portugal, and the Scandinavian countries), surveillance by sampling coastal phytoplankton is maintained in areas of significant shellfish production.[21] Increasing numbers of toxigenic *Dinophysis* or *Prorocentrum* trigger increased testing of shellfish for DSP toxins and closure of shellfish harvest if toxin levels exceed action levels. The principal

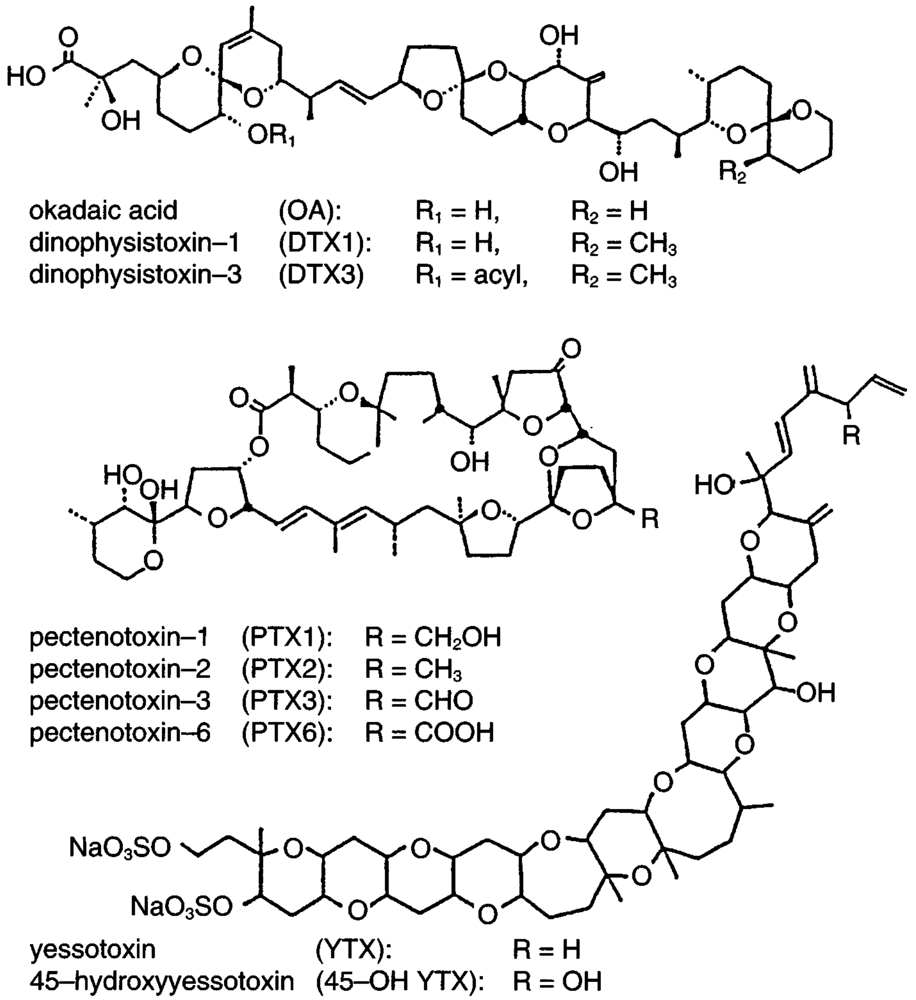

okadaic acid (OA): R₁ = H, R₂ = H
dinophysistoxin–1 (DTX1): R₁ = H, R₂ = CH₃
dinophysistoxin–3 (DTX3) R₁ = acyl, R₂ = CH₃
pectenotoxin–1 (PTX1): R = CH₂OH
pectenotoxin–2 (PTX2): R = CH₃
pectenotoxin–3 (PTX3): R = CHO
pectenotoxin–6 (PTX6): R = COOH
yessotoxin (YTX): R = H
45–hydroxyyessotoxin (45–OH YTX): R = OH
NaO₃SO
HO
OH
OR₁
R₂
R

Figure 54–2 Diarrhetic Shellfish Poisoning Toxins. *Source:* Reprinted with permission from T. Yasumoto, M. Murata, Y. Oshima, G. Matsumoto, and J. Clardy, Diarrhetic Shellfish Poisoning, in *Seafood Toxins*, E. Regalis, ed., pp. 207–214, © 1984, American Chemical Society.

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diarrheagenic toxins involved in DSP are OA and dinophysistoxins (DTX1-DTX3), but pectenotoxins and yessotoxin also may occur in toxic shellfish. A mouse bioassay for DSP based on the method originally described by Yasumoto[139] was used widely as the primary test for shellfish but was found to produce false-positive results due to contaminating fatty acids in shellfish digestive glands. A method that involves feeding tissue to white rats often is used.[51] A fluorometric HPLC method effectively identifies all of the toxins and their common derivatives when appropriate detection procedures are used.[7,60] In this procedure, digestive glands of shellfish are extracted with methanol, esterified to fluorescent esters with 9-anthryldiazomethane (ADAM), separated by HPLC, and monitored by fluorimetry. Toxin reference standards that are required for this method (OA, DTX1, DTX3) are now available commercially. Recently, useful modifications to improve sensitivity have been published.[95,138] The HPLC procedure easily detects concentrations below the legal limits used in Japan and Europe of 200 to 600 ng OA equivalent g^{-1} edible tissue.[95] A number of immunoassay procedures have been proposed and tested in recent years, and several test kits, primarily designed to identify OA, are available commercially in the US, Japan, and Canada.[92,138] New, improved procedures still appear in the literature.[20] There is disagreement concerning the usefulness of these and other proposed ELISA-type methods because of contrasting claims and experiences of too great a specificity on the one hand and insufficiently broad coverage on the other. A further complication is the occurrence of cross reactions between antisera for polyether toxins from different (defined) diseases (eg, ciguatoxins and okadaic acid), which is presented as an advantage or a disadvantage depending on the viewpoint of individual scientists.

54.2.3 Neurotoxic Shellfish Poisoning

Organisms and Their Toxins. NSP is caused by toxins produced by the unarmored dinoflagellates of the genus *Gymnodinium*. In the classical NSP outbreaks in the US, *G. breve* bloom in the Gulf of Mexico along the coast of Florida produced typical red tides and caused fish kills and toxic shellfish. Alternatively, the dinoflagellates may be entrained in the warm waters of the Gulf Stream and carried north off the US Atlantic coast. Blooms may be carried by eddies onto the Carolinas' beaches, producing red tides, fish kills, and toxic shellfish there.[132] Until recently, only one species, *G. breve*, was reportedly implicated, and outbreaks of NSP were confined to Florida and adjacent Atlantic states. However, in 1993, there was a large outbreak of shellfish poisoning in the North Island of New Zealand in which patients showed symptoms characteristic of NSP. The implicated dinoflagellate, though quite similar to G. *breve*, was determined to be a new species.[16,65] *Gymnodinium breve* produces a characteristic group of lipid-soluble polyether toxins, called brevetoxins, which act by opening Na+ ion channels, causing neurological symptoms. They are highly toxic to fish. The new species isolated from New Zealand waters produced closely related, but not identical, compounds. The unarmored *Gymnodinium* tend to be broken open by the pounding surf, which releases irritants that form an aerosol causing coughing and upper respiratory irritation in people on or near the beach.

The brevetoxins, shown in Figure 54–3, are methylated cyclic polyethers of two types, PbTx-1 type with a flexible backbone of 10 fused polyether rings and PbTx-2 type with a rigid ladder form of 11 rings.[94] They bind to a site in voltage-responsive sodium channels and essentially cause unintended firing of the neurons. Interestingly they seem to bind to the same sites as ciguatoxin.[8,94,120]

Characteristics of the Disease. The disease has been reported in individuals consuming oysters, clams, and other mollusks at the time of red tide occurrences in Florida and southern states along the US Atlantic coast.[8,132] In recent years, NSP has been reported in New Zealand.[65] Symptoms appear within minutes of consumption of toxic shellfish and generally subside within hours or, at most, a few days. Symptoms include tingling and numbness of the lips, tongue, throat, and perioral area; muscular aches and weakness; gastrointestinal upset; and dizziness. Some patients experience a reversal of hot and cold temperature sensation similar to that experienced in Ciguatera. This is not surprising since the brevetoxins responsible for NSP act by stimulating Na+ flow in voltage-gated sodium channels and attach to the same site as ciguatoxin. No fatalities and no sequelae have been reported. Concurrently with the shellfish-induced disease, there commonly are reports of upper respiratory irritation from individuals exposed to sea spray on or near beaches. This is a temporary and transient condition that does not involve the neurological symptoms of NSP.

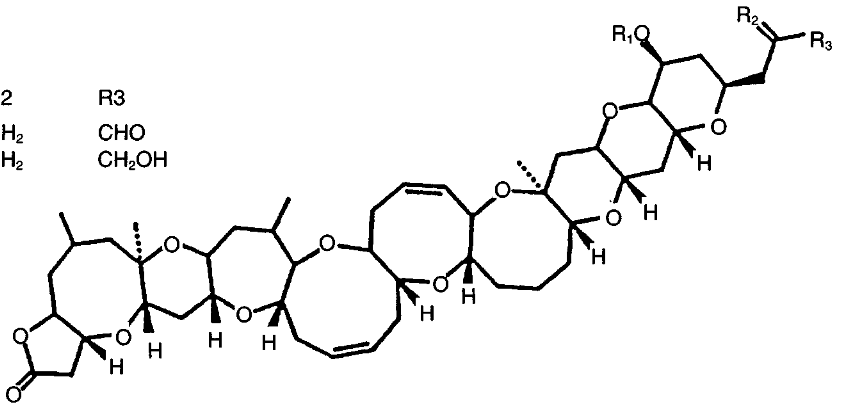
Until 1993, outbreaks were only reported from the US, where they were sparse in recent years because of quick responses by regulatory agencies to fish kills related to red tides and closures of shellfish harvest. However, the outbreak in New Zealand involved an excess of 180 cases.[16] This illustrates the suddenness with which shellfish poisoning can occur and the sensitivity of unprepared populations.

Food Vehicles and Transmission to the Consumer. Brevetoxins are potent killers of fish; therefore, finfish are not involved in human NSP, which is invariably caused by consumption of shellfish. Clams, oysters, and bay scallops have been implicated.[109,132] *G. breve* toxins have been reported to persist for 2 to 6 weeks in the oyster, *Crassostrea virginica*.[77]

Detection and Control. Occurrence of this disease is controlled primarily by surveillance based initially on identification of characteristic red tides in Florida and the Gulf and South Atlantic states of the US. Plankton sampling and analysis is undertaken and when *G. breve* exceed 5,000 organisms l^{-1} of sea water, shellfish harvesting is closed. In the US, harvesting is reopened when no detectable toxin is measurable in shellfish tissues by mouse assay, but practically this may be interpreted as equivalent to <20 MU 100 g^{-1} of tissue.[132] In New Zealand, where NSP-like toxicities occurred in 1993, phytoplankton analyses are carried out on a regular basis as part of their toxin surveillance system and toxic *Gymnodinium* are one of the target groups. Molluskan shellfish are also tested for toxic-

Type 1 Skeleton

		R1	R2	R3
Brevetoxin	1	H	CH_2	CHO
	7	H	CH_2	CH_2OH



Type 2 Skeleton

		R1	R2	R3
Brevetoxin	2	H	CH_2	CHO
	3	H	CH_2	CH_2OH
	5	$COCH_3$	CH_2	CHO
	6	H	CH_2	CHO (27,28—β—epoxide)
	8	H	O	CH_2Cl

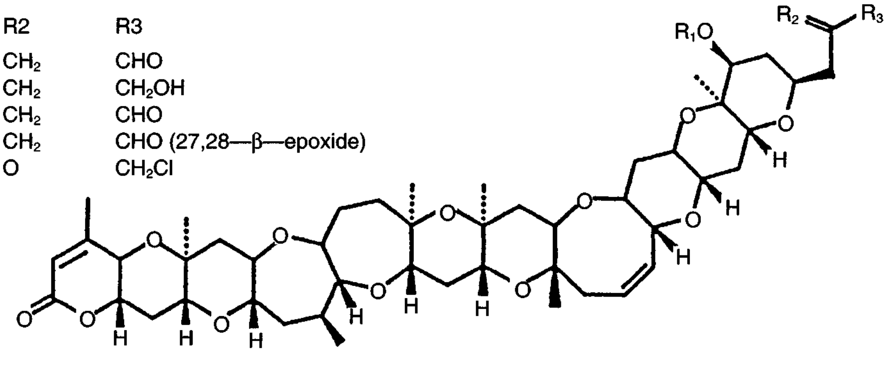


Figure 54–3 Neurotoxic Shellfish Poisoning Toxins. *Source*: Reprinted with permission from T. Motohiro, Biotoxins in Seafood, in *Quality Assurance in the Fishing Industry*, H.H. Huss, M. Jakobsen, and J. Liston, eds., pp. 243–258, © 1992, Elsevier Applied Science.

ity.[16] If a toxic bloom is apparent, shellfish are tested immediately. The official test is by a mouse bioassay of methanol extracts of shellfish tissue. Time of death of the mice, together with characteristic symptoms, are used to determine toxin levels. State or national authorities normally close shellfish harvesting when toxin is found in shellfish; closure is maintained until no toxin is identified in repeated samplings. The isolation and identification of the brevetoxins in recent years[8] and their availability from commercial sources have stimulated research on chemical, immunological, and tissue culture assays for these substances. The mouse neuroblastoma cell culture technique using veratridine and ouabain described above for PSP toxin assay also has been proposed as a method for brevetoxins. Researchers have reported detection limits of 2 ng ml^{-1} in a 4 to 6 hour test, which is both more sensitive and faster than the mouse test.[70] Immunoassays are complicated by the fact that the toxins fall into two categories and include multiple epitopes so that it is necessary to use at least two specific antibody preparations for effective total assay.[9,24] An ELISA method that worked well for sea water and phytoplankton was described.[9] Complications arise because of the appearance of different brevetoxin derivatives and it has been suggested that further research is needed before any method can be adopted for general use.[138] The close chemical and pharmacological similarity between brevetoxins and ciguatoxins makes development of a common test seem both likely and useful.

54.2.4 Amnesic Shellfish Poisoning

Organisms and Their Toxins. ASP is the most recently recognized of the major shellfish intoxications, being first described in an outbreak in Canada in 1987.[133] The responsible microorganism is a pennate diatom belonging to the genus *Nitzschia* (now *Pseudonitzschia*), and the primary toxin is domoic acid. For this reason the disease often is called domoic acid poisoning. Outbreaks or toxic diatom occurrences subsequently have been reported from the Pacific coast of the US and from New Zealand and Europe.[22,64,65]

The *Nitzschia* group is a very large one, composed principally of nontoxin producers. Toxic species now have been assigned to the genus *Pseudonitzschia* originally named for *P. australis* isolated from Argentinian waters. There are now three well-recognized species: *P. pungens* (*F multiseries*) (from the original Canadian outbreak),[121] *P. australis* (shown to be the toxin source in the US Pacific states outbreaks),[13] and *P. pseudodelicatissima* (from Bay of Fundy, Canada).[71] Recently, toxigenic *N. seriata*, now named *P. seriata*,[64] was identified from Danish waters and it is probable that other species will be described. Toxic *P. australis* has been identified in New Zealand and is probably fairly widespread in colder southern hemisphere waters. *P. pseudodelicatissima* caused extensive blooms in Danish, Swedish, and Norwegian waters, causing toxicities in mussels. The organism also has caused blooms in the western Baltic. Unlike the dinoflagellates that flourish in warmer waters, *Pseudonitzschia* blooms occur at low water temperatures of 10° C or below; these blooms often reach much higher densities in excess of 1 million cells l^{-1}.

Domoic acid (Figure 54–4) originally was recognized as a toxic component of the red seaweed *Chondria armata* and has been used as a antihelminthic medicine in Japan. A series of isomers designated isodomoic acid A through F have been identified in plankton and of these D, E, and F are involved as minor constituents in ASP.[96] All are tricarboxylic amino acids and act as an analog of glutamic acid, a neurotransmitter in the brain.[133] At low-dose levels, the toxin induces mainly gastrointestinal symptoms such as vomiting, diarrhea, and abdominal cramps, but at higher doses causes neurological signs including confusion, memory loss, and other symptoms; hence the designation amnesic poisoning. Domoic acid binds to glutamate receptors causing increasing firing of neurones and eventually rupture of cells and can cause death, particularly in elderly patients.[133]

Characteristics of the Disease. Amnesic shellfish poisoning caused outbreaks of disease among consumers of mussels in eastern Canada in 1987 and of razor clams in Washington state in 1991. Over 250 reports of illness were documented in the Canadian outbreak, and 107 fitted the strict case definition.[133] Only 12 cases were reported in the Washington state outbreak. A presumptive outbreak has been reported involving only two people who had eaten canned smoked oysters in South Korea in 1992.[99] Initial symptoms typically include vomiting, diarrhea, and abdominal cramps and (often severe) headache. Neurological symptoms develop somewhat later in more severe cases and involve confusion, memory loss, disorientation, dizziness, and even seizures or coma. The reported frequency of common symptoms from the large Canadian outbreak was nausea (77%), vomiting (76%), abdominal cramps (51%), headache (43%), diarrhea (42%), and memory loss (25%). Severity of the symptoms seems to be correlated with age; older people are more susceptible to memory loss. Mild cases show only gastroenteritis or mild neurological effects and recovery is rapid and uneventful. Severe cases can show persistent neurological dysfunction for several months. In a few cases, mainly involving older people, damage appeared to be permanent.[133] Onset of ASP symptoms may be as soon as 15 minutes after consuming toxic shellfish or may be delayed as long as 38 hours. There were four deaths in the Canadian outbreak, but none in Washington[136] or Korea. Nineteen patients in the Canadian episode were hospitalized showing confusion, disorientation, and inability to remember the recent past. Twelve of the patients requiring intensive care exhibited neurological abnormalities including coma, mutism, seizures, chewing and grimacing, uncontrolled crying or aggressiveness, profuse respiratory excretion, and unstable blood pressure or cardiac arrhythmias.[93] Examination of the brains of patients who died after eating contaminated mussels revealed severe damage to the hippocampus and amygdaloid nucleus.[131] Domoic acid, the toxin causing ASP, is a structural analog to the excitatory amino acid neurotransmitter glutamate and binds very strongly to a glutamate receptor; this is thought to cause hyperexcitation, producing the initial neurological symptoms and leading to degeneration of the hippocampus and other related sensitive components of the spinal cord.

The onset and severity of ASP is obviously dose-related, and patients in the Canadian outbreak have been estimated to have ingested 60 to 295 mg domoic acid based on concentrations of 31 to 128 mg 100 g^{-1} in mussel tissue. No symptoms were reported by individuals consuming up to 20 mg domoic acid (0.3 mg kg^{-1} body weight). It has been calculated that patients with mild symptoms ingested 60 to 110 mg (0.9 to 2.0 mg kg^{-1} body weight) and those with severe symptoms consumed 135 to 295 mg (1.9 to 4.2 mg kg^{-1}).[133] This contrasts with the Washington state outbreak in which all cases exhib-

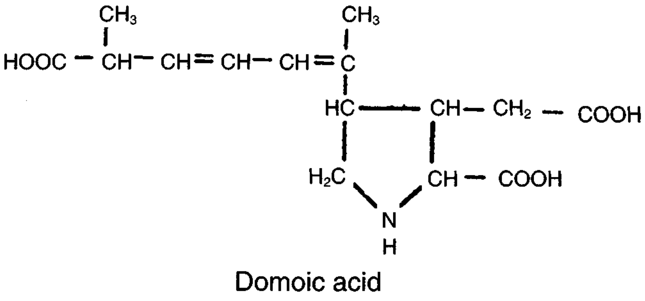


Figure 54–4 Amnesic Shellfish Poisoning Toxins. *Source:* Reprinted with permission from E.C.D. Todd, Domoic Acid and Amnesic Shellfish Poisoning—A Review, *Journal of Food Protection*, Vol. 56, pp. 69–83, © 1993, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

ited mild symptoms and ingested doses were only 0.05 to 0.39 mg kg^{-1} but unaffected persons ingested up to 0.28 mg kg^{-1}. No estimates were available for the Korean incidents.

Food Vehicles and Transmission to the Consumer. Domoic acid originally was associated with mussels in the Prince Edward Island ASP outbreak in 1987, but in subsequent years was recovered from the digestive glands of clams and scallops off Georges bank and the coast of Maine and from horse mussels and soft shell clams in Nova Scotia and New Brunswick.[133] In the US west coast incidents, domoic acid was identified in Californian anchovies (the source of illness and death of pelicans and cormorants) and later at low levels in mussels, and stone and rock crab viscera.[135,137] However, the toxin was detected at dangerous levels in razor clams in Washington state, causing several human ASP cases, and in Dungeness crab viscera.[22,44] Not surprisingly, the toxin later was reported from British Columbia, the Canadian province adjacent to Washington, from the same animals. Although the consequences of the sudden appearance of ASP in western North America were less severe in terms of human illness and death than the original Canadian episode, the occurrence deserves to be noted as a further warning of the risk presented by unexpected marine toxic incidents. The rapid and effective response by public health and other agencies in closing shellfish harvest and fish processing of anchovies was possible because of the existence of the PSP surveillance system in the affected states and provinces.[84] Domoic acid contamination of shellfish has been reported recently in New Zealand as a result of a routine screening program, with highest concentration and longest persistence in scallop viscera. *Pseudonitzschia australis* has been associated with the contamination, but a possibility that other benthic diatoms may be involved cannot be excluded.[66] This further emphasizes the need for alertness by regulatory and health authorities in coastal countries, particularly in northern and southern cold water regions, to the possibility of domoic acid contamination of shellfish. The foresight of authorities in New Zealand probably prevented human illness.

Detection and Control. Initially, ASP assays were conducted on mice using extracts prepared according to the PSP protocol. In positive cases, mice showed an involuntary scratching of both shoulders within 4 hours (typically 7 to 21 minutes) after IP injection, followed by increasingly uncoordinated movements and seizures. Death usually came within a few hours.[133] Unfortunately, mouse response was positive only for shellfish tissue levels of >40 µg domoic acid g^{-1} wet weight, well above the regulatory level of 20 µg g^{-1}. Consequently, an HPLC method has been adopted as the method of choice for analyzing shellfish samples for domoic acid.[59,133] This has an effective limit of 1 µg g^{-1} of tissue but lower levels are obtainable by modifying extraction methods.[95] Analytical methods used in different countries are detailed in a recent publication of IOC.[96] Recently, an ELISA type immunoassay has been described using standard PSP type extracts with lower limit of detection below 0.5 µg g^{-1}.[114] The authors indicate that the test would be suitable for field testing.

54.3 FISH POISONING

Human poisonings from toxic fish differ from shellfish poisoning in a number of ways. They generally are caused by consumption of flesh from a single animal, so outbreaks involve a limited number of people, while shellfish incidents usually involve consumption of several toxic animals and outbreaks may be large. The mobility of fish makes identification of toxic focii difficult except in the case of scombroid poisoning, which develops in dead animals after capture, while beds of toxic shellfish are readily located. The origin of fish toxins also is more varied than shellfish toxins involving bacteria as well as dinoflagellates. It is noteworthy that human poisoning has occurred as a result of eating fish contaminated with PSP toxins,[67] and marine bird and mammal intoxications have been associated with

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consumption of fish that have fed on toxic (PSP and ASP) phytoplankton.[133] There is good reason to believe that in these cases toxin was derived from toxic plankton residues in the fish intestine rather than the muscle tissues normally eaten by human consumers. The fish poisonings discussed in this section are caused by eating toxic muscle tissue, though in fish causing ciguatera and puffer fish poisoning, viscera can be a potent source of toxin and may physically contaminate edible flesh.

Because of the dispersed distribution of toxic fish in the environment, there is very limited regulation of harvest, and human protection depends mainly on general warnings issued periodically to the industry and consumers. However, landing, sale, and distribution of particularly dangerous species may be prohibited in particular countries or regions either seasonally or on a permanent basis and there are special regulations regarding puffer fish in Japan.

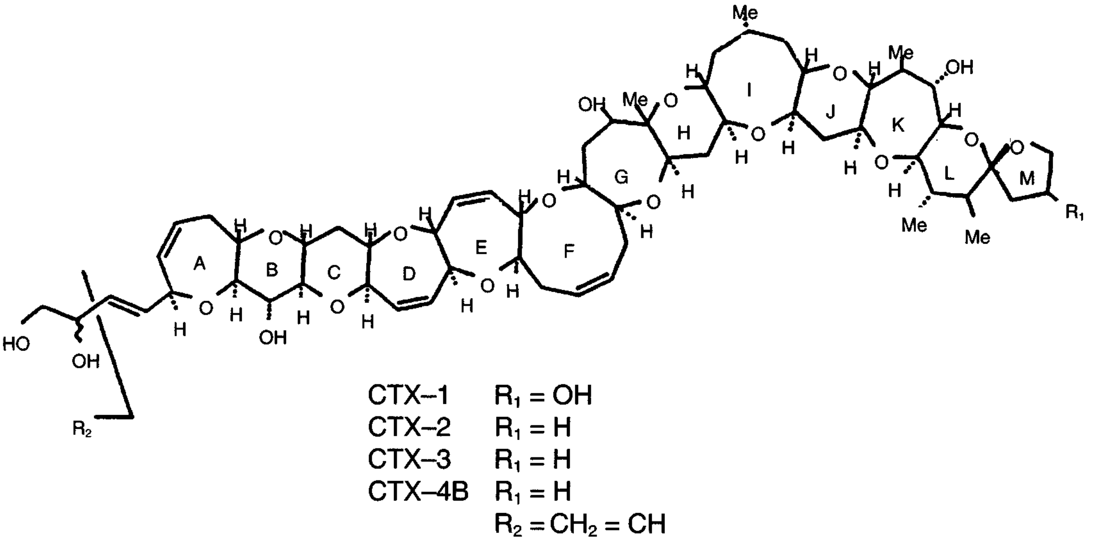
54.3.1 Ciguatera

Organisms and Their Toxins. Ciguatera is caused by ingestion of tropical or subtropical fish species that contain ciguatoxin and related toxins originating from the dinoflagellate *Gambierdiscus toxicus.* This toxigenic microalga is benthic and commonly is isolated from macroalgae growing on or near coral reefs. Recently, the organism has been isolated from seaweed growing on a sandy bottom in tropical Australian waters.[62] Herbivorous fish and invertebrates grazing on the reef vegetation or on benthic macroalgae ingest *G. toxicus* when present and accumulate toxin in the alimentary canal and in the tissues. The herbivores frequently are prey of carnivorous species, which may then become toxic by toxin transfer. Secondary carnivore-carnivore predation leads to progressive accumulation of toxin continuing through the life cycle so that large fish are typically more poisonous than smaller fish of the same species.[33]

Gambierdiscus toxicus is a slow-growing dinoflagellate that is an epiphyte of other macroalgae, does not produce cysts, and has been reported to be capable of growth at water temperatures of 20° C to 34° C, in salinities from 2.5% to 4%, and at depths from 0.1 m to 30 m.[62] It is a characteristic of ciguatera that the occurrence of toxic fish on reefs is variable. Some reef areas tend to be more frequently implicated than others as toxin sources, but there is a degree of unpredictability concerning toxic sites that suggests the growth of *Gambierdiscus* is influenced by changing environmental conditions.[62] It has been reported that population density of *G. toxicus* in nature increases and decreases rapidly without regard to seasonal changes. There are indications that degradation of reef integrity, sometimes due to human activity, results in upsurges in ciguatera but the mechanism of these effects is not understood.[101]

The major toxins in Pacific fish causing ciguatera appear to be ciguatoxins, but other toxins may be concurrently present including scaritoxin and maitotoxin. Yasumoto et al. provide a useful overview of these toxins;[138] their structures are shown in Figure 54–5. The ciguatoxins are lipid-soluble polyethers believed to derive from toxins (sometimes called gambiertoxins) that are produced by *G. toxicus* as a result of biotransformation occurring during transfer to fish or within fish tissues.[62] At least six congeners labeled CTX 1-4, 4A, and 4B have been identified; CTX1, or simply CTX, the ciguatoxin originally identified by Schauer's group,[107] is the principal toxin encountered in cases of ciguatera. These toxins are potent activators of voltage-dependent sodium channels in nerve cells and produce essentially neurological symptoms in humans. Scaritoxin is as yet uncharacterized but could be a gambiertoxin derivative. Maitotoxin, produced in large quantities by *G. toxicus*, is a polyether toxin that activates Ca^{2+} release influencing muscle contraction. It is found particularly in small herbivorous fish.[138] This toxin has powerful effects on mice (LD_{50} for mice by IP, 50 ng kg^{-1}), but its toxicity for humans has been questioned. Other toxins identified in ciguateric fish include OA and palyotoxin; these are obviously derived from sources other than *G. toxicus* and it is unclear whether they participate in the ciguatera syndrome.[53,54] A further complication in toxin identity and function arises from the fact that Caribbean outbreaks of ciguatera show a different balance of symptoms than Pacific cases, suggesting that different toxins may be involved. This is supported by the poor success of an immunochemical test that worked well for ciguatera toxins of Pacific fish in identifying toxic Caribbean fish;[91] nevertheless, polyether type toxins are indicated in all cases.[62]

Characteristics of the Disease. This disease is a clinical syndrome consisting of gastrointestinal and neurological symptoms occurring after consumption of ciguatoxic fish. The gastrointestinal symptoms appear 3 to 5 hours after ingestion of ciguatoxic fish and are soon resolved, while neurological symptoms that begin 12 to 18 hours after consumption may be



CTX–1 $R_1 = OH$
CTX–2 $R_1 = H$
CTX–3 $R_1 = H$
CTX–4B $R_1 = H$
$R_2 = CH_2 = CH$

Figure 54–5 Ciguatera Toxins. *Source:* Reprinted with permission from R.J. Lewis and M.J. Holmes, Mini Review, Origin and Transfer of Toxins Involved in Ciguatera, *Comparative Biochemistry & Physiology,* Vol. 106 C, pp. 615–628, © 1993, Elsevier Applied Science.

moderate to severe and normally last for 1 to 82 days. A wide range of symptoms has been reported, including nausea; vomiting; diarrhea; prickling of the lips, tongue, and throat; numbness; headache; arthralgia; erythism; dizziness; cyanosis; insomnia; prostration; ataxia; and myalgia. There is severe pain, particularly in the arms, legs, and eyes, vision disturbances, and teeth feel loose. Skin disorders appear, including erythema, maculopapular eruption, blisters, stinging sensation, and loss of hair and nails. The neurotoxic symptoms are most pronounced in severe cases and often include the characteristic reversal of temperature perception where cold is perceived as hot ("dry ice" or "electric shock" sensation) and hot objects are perceived as cold. General motor incoordination, paralysis, and convulsion progressively become worse, sometimes progressing to coma and death. The fatality rate is, however, low and the majority of cases are mild and resolve within a few days or weeks; in more severe cases, recovery can be slow, extending over months or years.[58,75,134] Recurrence of some symptoms is not uncommon, stimulated by alcohol consumption or sometimes by eating fish. The variety of symptoms reported suggests that more than one toxin is involved.[53] Treatment of ciguatera is designed to relieve the more severe symptoms; intravenous mannitol infusion recently has become the treatment of choice in acute cases; atropine may be used for symptomatic bradyarrhythmias. A variety of drugs has been tried for management of chronic symptoms including amytriptyline and tocainide, with variable success in individual patients.[58,90,134]

Ciguatera is reported principally from tropical regions, particularly island communities of the tropical Pacific and Caribbean oceans, but also from Australia, India, and other countries with warm-water coastlines. Incidence of cases in the island communities in which fish is an important component of the diet is high with estimates of several events in the lifetime of residents. Actual incidence was calculated to be 730/100,000 population per year (1970s) in the Virgin Islands,[75] 234.9/100,000 population in the Marshall Islands (1980s),[101] and 50 to 500/100,000 in Miami. Regalis, in 1984,[97] reported an estimate of 50,000 cases/year globally while a more recent estimate (1993) suggests in excess of 25,000 cases/year.[62] Obviously the true incidence of ciguatera is very difficult to determine because of the wide distribution of cases and the absence of good reporting systems or records in many locales. There has been a surge in interest in the disease because of numerous incidents involving tourists in island resorts and because of an increase in reports of ciguatera in nontropical countries, caused by imported fish caught in areas of ciguatera incidence.[46]

There is still some uncertainty concerning the diagnosis of ciguatera and the role of various toxins in its pathology. The principal toxins identified in ciguatoxic fish include ciguatoxins 1, 2, and 3, scaritoxin, and maitotoxin, but okadaic acid and even palytoxin as well as other water-soluble toxins have been reported in some cases.[23,24,48] Ciguatoxins are dominant in ciguateric fish from the Pacific regions[62] and probably also Caribbean species, and their known ability to bind to sodium channels and promote activation can account for much of the neurological effects observed in patients. It has been proposed that ciguatoxin causes a nerve conduction block after the initial neural stimulation.[32] Scaritoxin is another lipid-soluble toxin that has a depolarizing action on excitable membranes and also depresses oxidative processes in rat brain.[61] This toxin was identified as the cause of delayed ataxia in a case involving ciguateric parrotfish.[17] OA has been suggested as a factor accounting for the increased incidence of gastrointestinal signs in ciguatera caused by Caribbean fish as compared with Pacific species.[31] Palyotoxin is a polyether toxin and causes clinically distinguishable symptoms from ciguatera, but a mild palyotoxin episode might simulate ciguatera.[54,62] Recent studies of the application of a new commercially available immune bead test for ciguatera that appears to pick up a variety of polyether toxins has pointed toward a rather general incidence of polyethers, including brevetoxins in fish from ciguateric areas, but dominantly OA. This brings into question the specificity of ciguatera as a single definable disease or its existence as a useful description of a somewhat variable syndrome.[91,92] It also is possible that the identification of *G. toxicus* as the sole origin of ciguatera toxins is not correct; other dinoflagellates have been implicated in the past,[62] and recently the cyanobacterium *Trichodesmium erythraeum* has been shown to produce ciguatera toxins.[24] Whether a single origin or not, the condition is of widespread occurrence in the tropics and has both important human and economic consequences for individuals and society.

Food Vehicles and Transmission to the Consumer. More than 400 species of fish have been listed as potentially ciguateric.[33,39] The fish most commonly implicated in outbreaks reported to the U.S. Centers for Disease Control and Prevention (CDC) 1978 to 1987 were amberjack (*Seriola*), snapper (*Lutjanidae*), grouper (*Serranidae*), goatfish (*Mullidae*), Jacks (*Carangidae*), and barracuda (*Sphyrenidae*).[46] Most frequently involved elsewhere were *Scombroididae* in Australia, *Sphyrenidae* in Fiji, and *Acanthuridae* and *Serranidae* in French Polynesia.[33,58,134] The possibility of ingesting a ciguateric fish is quite high for fish eaters in certain areas of the world, specifically the islands of the tropical Pacific, the Caribbean and adjacent countries, coastal India and Asia, Australia, and anywhere adjacent to tropical reefs. Indeed, the ubiquity of toxic fish in particular locations, and the necessity of fish as an accessible and affordable source of animal protein for local inhabitants, make repeated experiences of ciguatera almost inevitable. Because the disease rarely is fatal and ultimately is self-resolving in most cases, there is a low level of attention by local regulatory authorities, although sale of known dangerous species is banned in some cases (eg, groupers, snapper, barracuda, and surgeonfish in French Polynesia[33] and barracuda in Florida). The risk for the traveler or tourist can be equally high, and government agencies in favorite tourist locations (eg, Caribbean Islands) as well as some international organizations and the tourist industry attempt to provide information and warnings to incoming visitors.

Detection and Control. Fish poisoning due to ciguatera toxins obviously cannot be dealt with like shellfish intoxications. Toxin accumulation occurs through a food web of wide distribution and involving mobile components. Although it is possible in isolated island groups in the south Pacific to track toxin sources to a particular reef or even parts of a reef, the task becomes more difficult when the number of adjacent islands is large or the fishing area is extensive. Tracking large, mobile, predatory fish to a toxin source is even more improbable and not particularly useful for any practicable control. Unfortunately, at the present time, toxic fish usually are identified when they cause human illness. Some control is achieved by banning the landing or presentation for sale of certain high-risk species in regions where ciguatera occurs (eg, barracuda in Florida; groupers, barracuda, snappers, and surgeonfish in French Polynesia; amberjack in Hawaii),[33] but the free movement of most species in international commerce makes the deliberate or accidental circumvention of such rules almost inevitable, and so cases of ciguatera continue to occur in tropical island communities and widely among importing countries. Truly effective control requires that toxic fish be recognized and intercepted at some point between capture and the consumer's plate. This requires an effective and preferably simple, inexpensive, and a nondestructive test for toxicity that can be performed at point of capture, landing, or sale.[46] Such a test could be applied individually to each of the larger fish of likely ciguateric species that usually constitute the major problem and normally are caught by hook and line.

A variety of animals were used to test for ciguatoxic fish prior to the isolation and chemical identification of CTX by Scheuer and his group in 1967.[107] Thereafter, a more definitive mouse assay was used for this toxin and its analogs. Toxins are extracted from fish tissues using lipid solvents, such as methanol, diluted, and injected IP into mice. Typical symptoms develop, and death occurs over a 24 to 48 hour period. Symptoms include diarrhea within 15 minutes, dyspnea and cyanosis later, penis erection, and dyspnea-derived convulsion before death.[130] Body temperature also may be depressed. Quantitative assay may be obtained by using dose survival time data for pure toxin. One MU is equivalent to 7 ng CTX, and as little as 10 MU have been reported to cause poisoning.

Fluorescent esters may be made of CTX, and this has formed the basis of a liquid chromatographic method for estimating some, but not all, of the CTX toxin group.[138] The method worked well with carnivorous fish; however, there is a problem in detecting low toxin levels that can cause ciguatera (eg, 35 ng 100 g^{-1}), especially in herbivores in which toxins lacking the reactive group to produce fluorescent esters are more abundant.

The cell assay system using mouse neuroblastoma cells described earlier for analysis of saxitoxins and brevetoxins can be used for ciguatoxins. Two groups of scientists have shown this method to be highly sensitive for CTX toxins, detecting from 0.1 to 5 nmole CTX kg^{-1} in toxic flesh fish.[70,138] The method was reported to correspond well with mouse bioassay (though much more sensitive) and to be indifferent to the regional source of the ciguateric fish, whether Pacific or Caribbean.

A number of immunoassay procedures for ciguatera have been described in the scientific literature,[91] and some are under collaborative study. Perhaps the longest series of studies are those of Hokama and his colleagues culminating most recently in the commercially available Ciguakit based on a monoclonal antibody specific to ciguatoxin, OA, and a fragment of OA.[40–42] This system thus can be used to detect toxins of ciguatera and DSP. The method has reportedly worked well for ciguateric Pacific fish and DSP shellfish in Europe,[92] but has been less successful with ciguateric fish from the Caribbean. Another series of studies of immunoassay procedures by a group at the University of Miami has culminated in an ELISA system based on two antibodies that are targeted at brevetoxins and ciguatoxins but do not cross react with other polyether toxins such as OA and dinophysistotoxins.[9] The system has been shown to work well for dinoflagellates and isolated toxins but not for fish or shellfish.

54.3.2 Scombroid Fish Poisoning

Organisms and Their Toxins. This disease differs from all other natural fish poisonings because the toxin is produced in the fish post mortem and thus is potentially controllable. Moreover, bacteria, probably of human origin because they are not natural inhabitants of the oceans, are the most probable cause of the toxicity, which appears to be histamine poisoning though other amines may be involved.[52] Originally, it was thought that the problem only occurred in fish of the *Scombridae* (mainly tunas and mackerels)—hence the disease name—but subsequently, it became clear that a much wider range of fish is involved, including many pelagic, rapid-swimming species with relatively high natural tissue levels of free (unbound) histidine; a common and nutritionally desirable amino acid. Histamine is produced in such fish post mortem as a result of growth of bacteria that produce histidine decarboxylase. When fish are held at cool temperatures (<10° C), the rate of production of histamine is slow and the concentration at the point of rejection for spoilage is normally below the danger level of 50 mg histidine 100 g^{-1} fish tissue.[28,87] This corresponds to growth of psychrotrophic spoilage bacteria that are slow histamine producers. Storage of fish at higher temperatures (>15° C) results in more rapid production of histamine with levels of >100mg 100g^{-1} reached before spoilage rejection.[10] In this case, the bacteria dominating the fish microflora are mesophilic and include strong histamine producers.[10,87] Arrhenius plots of histamine formation and—separately—of organoleptic spoilage in stored scombrotoxic fish show deviation from linearity between 5° C and 10° C, confirming involvement of distinct psychrophilic and mesophilic bacteria below and above this temperature range.[10,63] The bacteria most commonly implicated in scombroid poisoning are members of the Enterobacteriaceae; specifically, *Morganella morganii*, *Klebsiella pneumoniae*, and *Hafnia alvei*, but *Clostridium perfringens* also may be involved in some cases.[130] These bacteria grow rapidly at temperatures above 20° C and characteristically produce active histidine decarboxylase resulting in accumulation of significant levels of histamine in the tissues of dark fleshed fish. They are not normally part of the microflora of living fish, except for *C. perfringens*, which is found in the intestine of fish caught in inshore waters. Thus, it is probable that these bacteria are contaminants from handling the fish after its capture. Clearly, the fish must be exposed to warm temperatures after contamination for the few hours necessary for histamine to be produced and accumulate to a toxic level if scombroid poisoning is to occur in individuals eating the fish. In most cases, fish involved in such poisonings have been found to have levels of histamine >100 mg 100 g^{-1} of muscle tissue; however, there have been reports of fish with much lower histamine levels causing poisoning. It should be noted that the normal course of spoilage in pelagic dark-fleshed fish held under refrigeration temperatures (<5° C) usually results in the production of histamine, but at levels below those causing illness (eg, <20mg 100 g^{-1} of fish tissue).

The substance primarily implicated in scombroid poisoning is histamine, and the initial symptoms, which to some extent mimic an allergic response, support this conclusion.[102] The amount of histamine apparently ingested by patients showing the symptoms sometimes is less than the amount shown to be necessary by experimental administration of histamine to volunteers. Japanese scientists initially identified saurine, a substance with histamine-like properties, as the cause of the disease, but this was shown to be an artifact of the separation technique.[26,52,111] The decarboxylase activity of bacteria growing on the fish post mortem results in accumulation of other amines, including cadaverine and putrescene, which have been shown experimentally to enhance the toxic effect of histamine. Specifically, it has been proposed that there is partial inhibition of the natural histaminase activity in the alimentary canal or interference in its function. An alternative proposal by one group of researchers, based in part on human volunteer studies, is that some component in scombrotoxic fish, perhaps an algal toxin, causes human mast cells in situ to produce histamine, thus giving rise to the characteristic symptoms.[18,45] These findings have been contradicted by other observations that indicate histamine is not produced endogenously by this mechanism in patients,[76] and the view that currently prevails is that scombroid poisoning is a result of histamine accumulated in fish as a consequence of temperature control failure at some point between capture and consumption.

Characteristics of the Disease. This is a disease of truly worldwide incidence, although it is more frequently associated with fish from warm-water regions than temperate species. As noted earlier, it differs from the other seafood intoxications in being caused by the activity of bacteria accumulating toxic histamine (and perhaps other active substances) as a consequence of metabolic activity in or on dead fish after capture. Symptoms appear a few minutes to an hour after consuming the toxic fish and initially resemble anaphylaxis. They include a metallic, sharp, or peppery taste; intense headache; dizziness; nausea; vomiting; facial swelling and flushing; epigastric pain; throbbing of carotid and temporal vessels; rapid and weak pulse; burning throat; thirst; difficulty swallowing; edema; itchy skin; audible wheezing; and diarrhea.[102] In most cases, the symptoms disappear quickly, with complete recovery in 10 to 12 hours. Specific treatment usually is unnecessary but oral antihistamines may be beneficial in severe cases.[102] Fatalities are unusual and usually due to other, unrelated contributing causes.[130]

Scombroid fish poisoning is the principal cause of fish poisoning in the US and is commonly reported from many other countries. Because the symptoms are so transient and, in most cases, so mild, this poisoning frequently is not reported to public health authorities, making it difficult to obtain realistic estimates of the true incidence. Improvements in handling of tuna destined for canning and screening of raw fish for histamine content by commercial canners virtually have eliminated scombroid poisoning due to canned tuna, but fresh and frozen tuna still cause poisoning incidents. The incidence of reported cases of scombroid poisoning in various countries until 1982 is listed in Taylor.[130] He notes that statistics are fragmentary for most countries but gives numbers for Japan, the UK, and the US. Early Japanese outbreaks were characterized by high case numbers; during 1951–1954, there were 14 outbreaks with 1,215 cases (including 1 outbreak with 700 cases), while during 1970–1980, there were 42 outbreaks involving 4,122 cases (with 1 outbreak of 2,656 cases). Taylor reports 136 outbreaks in the UK for 1976–1982, with a total of 439 cases indicating much smaller sized outbreaks. Small outbreaks were also reported in the US, where between 1968 and 1977, 55 reported outbreaks involved 516 cases. A later report for the US[46] indicated 157 cases with 757 cases in the period 1978–1980, confirming a continuing trend for small-sized outbreaks. Scombroid poisoning has now been reported from virtually every country in the world and, though reliable estimates of total world incidence are not available, cases annually must total in the thousands.

Food Vehicles and Transmission to the Consumer. The number of fish species reportedly involved in scombroid fish poisoning continues to increase as reports appear from different countries around the world. The majority of incidents involve scombroid fish, particularly mackerels and tunas (including bonito) and nonscombroids such as dolphinfish or mahi mahi (*Coryphaena hippuris*), sardines, anchovies, pilchard, herrings, Atlantic bluefish (*Pomatomas saltatrix*), marlins, and yellowtail (*Carangidae*).[80,132,134] More generally, marine pelagic fish with characteristically dark meat are likely to be involved. This is truly a disease of worldwide incidence, but the risk may be highest for fish caught in warm waters and exposed to high ambient temperatures after capture. Processes that involve heating the fish to temperatures that promote the growth of mesophilic bacteria such as smoking or sun drying can cause accumulation of histamine to dangerous levels if proper controls are lacking. Moreover, since histamine is not destroyed by heat processes used in canning, canned fish can be a vehicle for scombroid poisoning. Thus the risk to consumers depends on both the species of fish and the quality of handling and processing. Risk could in theory be eliminated by testing all fish of incriminated species for histamine since several analytical procedures are available for this substance. Obviously, this is impractical and usually only fish in batch-type operations such as tuna for canning or species likely or known to have been mishandled are tested.

Detection and Control. The analytical procedures for scombroid fish poisoning are limited to methods for histamine because no other compound has been demonstrated to indicate toxicity convincingly. There are a number of reliable analytical methods for histamine but the most widely used is the fluorometric procedure described in AOAC.[6]

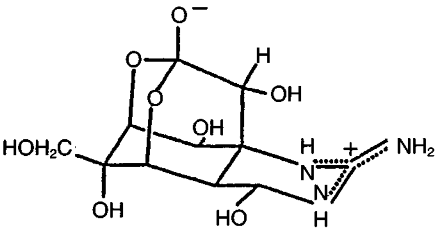
Control of scombroid poisoning is dependent on proper handling of fish after capture and during storage, processing and transportation, and the interception and removal from the food supply of fish with high histamine content (>5 mg 100 g^{-1} fish) before it reaches the consumer. The usually mild and self-resolving nature of scombroid poisoning precludes large investment in routine analysis of potentially toxic fish. It is, therefore, likely to be much more productive to emphasize the importance of rapid chilling of fish after death, maintenance of low temperatures during storage and, where possible, during processing. All fish should be held as close to 0° C as possible to limit the rate of spoilage as a matter of good commercial practice. Histamine development to levels causing symptoms is due to the growth of mesophilic bacteria producing copious amounts of amino acid decarboxylases in or on fish, which have high levels of free histidine. This clearly indicates that the flesh temperature of the fish has exceeded 15° C over a period of at least 12 hours. For large fish, such as tuna, caught in warm surface waters and with a body temperature often exceeding 25° C, this would not be an unusual situation unless specific measures are taken immediately after capture to cool the fish quickly. The time necessary to cool single large fish or bulked smaller species with inadequate chilling procedures (eg, top icing) is frequently underestimated. Moreover, intermittent inadvertent reheating of fish during transport or processing (eg, smoking) can create conditions for bacteria and amino acid decarboxylases to accumulate or become active. Some enzyme activity has been shown to continue during cool storage and may be sufficient to increase histamine above toxic levels.[63]

54.3.3 Puffer Fish (Fugu) Poisoning

Organisms and Their Toxins. Puffer fish poisoning results from the presence of tetrodotoxin in the tissues of puffer fish.[78] The name puffer fish was given because these fish characteristically swallow air or water to inflate their spiny bodies when threatened, probably as a disincentive to predators. Certain species are considered a delicacy in Japan and other Asian countries. The toxin is not consistently present in fish, but is most concentrated in the liver and other internal organs and unevenly present, in lesser amounts, in the muscle. Tetrodotoxin (TTX) is most prevalent in and near the gonads, particularly during the spawning season.[134] TTX also has been isolated from a number of other animals including goby fish, newts, frogs, octopus, ivory shell, trumpet shell, starfish, flatworms, various crabs, and gastropods, some of which have been involved in human poisonings.[78,134,141] Puffer fish cultured under controlled conditions are nontoxic but can be made toxic by feeding them liver from toxic fish, suggesting a food chain origin for the toxin;[103] however, bacteria that produce TTX and dehydrotetrodotoxin have been isolated from the skin and intestine of puffer fish and other sources.[85,122,140,142] The toxigenic bacteria originally were identified as *Pseudomonas* and *Vibrio* spp., particularly *V. alginolyticus*, a common bacterium on fish and shellfish.[113] Tamplin[127] lists 21 bacterial species reported to produce tetrodotoxins, including *Vibrios* (9), *Alteromonas* (5), *Aeromonas* (2), *Plesiomonas* (1), *Pseudomonas* (1), and *Escherichia coli* (1).[127] More recently, three of the toxigenic strains isolated from red algae and puffer fish were identified definitively as *Listonella pelagia* (*V. pelagius*), *Alteromonas tetraodonis*, and *Shewanella alga.*[112] *Vibrio* spp. typically occur in the intestine of marine fish, and it has been shown to dominate the microflora of puffer fish (*Fugu niphobles*) when the fish were reared in water at 29° C. It has been proposed, therefore, that TTX-producing bacteria growing in the intestine of puffer fish are the source of the toxin in these fish and more generally of toxin in the other TTX-containing animals.[122] Unfortunately, even if this should be proved, little can be done to prevent the accumulation of toxin in wild fish. TTX, though unlike STX chemically (Figure 54–6), is also a Na+ channel blocker and has an equally high toxicity for humans (2 mg may be lethal) and unfortunately continues to claim victims in Japan and elsewhere.[88,138]

Characteristics of the Disease. This disease is reported mostly from Japan and, less frequently, other Southeast Asian countries;[73,132] however, it also has been reported from the US.[46] The symptoms of puffer fish poisoning are similar to those from PSP and, though quite different chemically, the two classes of toxin act in the same way to block transmission of Na^+ ion in nerve cells. Onset is rapid (within minutes of consumption of toxic flesh), with tingling of lips, tongue, and fingers leading to paresthesis of extremities. Nausea and vomiting are also early symptoms. In severe cases, there is progressive paralysis, and the patient may die from asphyxiation due to respiratory paralysis. Severity of the disease is dependent on the dose of TTX absorbed. Dose levels are remarkably similar to those for PSP, with 1 to 4 mg being lethal. Treatment is supportive with gastric lavage and respiratory assistance the principal recommended actions.[134] The disease causes a variable number of cases in Japan each year where the toxic species of puffer (or fugu) are considered a delicacy. The fish are prepared and served in special restaurants by specifically trained fugu cooks who seek to present only the portions of the fish with no or minimal toxin; nevertheless, each year consumers of Fugu become sick and some die.[88] There is confusion among some fishermen and consumers regarding which puffers are toxic or when potentially toxic species become toxic. Even in the US the Northern Puffer (*Sphoeroides*) was declared a nontoxic species in an article published in 1988 despite the fact that seven cases, including three fatalities, were reported in Florida between 1951 and 1974.[46] The common puffer fish, *Arothron hispidus*, has been implicated in at least seven fatalities in Hawaii in recent years, and outbreaks still occur in other Pacific countries.[141] It has been reported that puffer fish raised by culture are nontoxic,[103] which raises prospects for a safe fugu product. Similarly, an understanding of the role of bacteria in producing the toxin particularly in view of the observations by Gallagher and Birkbeck[30] concerning metabolic control of tetrodotoxin production could be used to modify aquaculture procedures to ensure toxin-free fish. However, it is difficult to see how this knowledge could affect the safety of ocean-caught fish, and wild puffer fish must be assumed to represent a high risk of illness and death for the uninformed consumer.

Food Vehicles and Transmission to the Consumer. Though TTX has been found in a range of marine and other animals, the risk to consumers seems to be mainly from puffer fish. Different species of puffer fish vary in their toxicity. Particularly toxic species include *Fugu niphobles*, *F. poecilonotum*, *F. vermiculare*, and *F. pardale.*[79] The dangers of eating such fish are generally well recognized by fish eaters and regulators in Japan and other countries in Southeast Asia and some degree of risk



ANHYDROTETRODOTOXIN

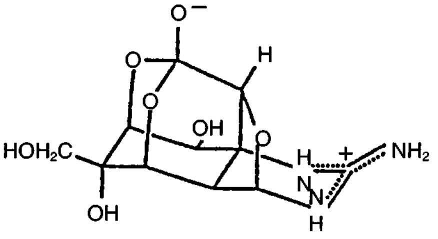


Figure 54–6 Puffer Fish Poisoning Toxins. *Source:* Reprinted with permission from T. Motohiro, Biotoxins in Seafood, in *Quality Assurance in the Fishing Industry*, H.H. Huss, M. Jakobsen, and J. Liston, eds., pp. 243–258, © 1992, Elsevier Applied Science.

is accepted by fugu eaters to achieve the "fugu experience."[73,134] There is little risk to the general fish-eating public because the toxic puffer fish species are not widely marketed.

Detection and Control. Control of puffer fish poisoning requires strict surveillance of fish destined for fugu establishments, and now in several countries (eg, Japan and the US), the authorities require assurance that the fish is nontoxic. This requires regular toxin testing traditionally done by a mouse assay using similar procedures to those described for PSP. There is a high fatality rate associated with puffer fish poisoning, and this is reflected by rapid death of test mice. One MU is the amount of TTX that will kill a male mouse of the Japanese ddY strain weighing 20 g in 30 minutes and is equivalent to 220 ng TTX: it has been estimated that 10,000 MU cause death in a human.[79] A fluorometric TTX analyzer has been developed in Japan.[138] This system uses extracts prepared by boiling fish tissues with .02 N acetic acid and passing the centrifuged, membrane-filtered (<10,000), suitably cooled and diluted extract through *LC* column to separate TTX and its congeners. The column is eluted with heptafluorobutyric acid and heated with NaOH and read in a fluorometer. The method has a minimum detection level of 5 ng per injection and is simple and effective for routine monitoring.

Obviously, the mouse neuroblastoma cell method described for STX testing works equally well as an assay for TTX because both toxins are detected by their Na^+ channel-blocking effect, which antagonizes the effects of ouabain and veratridine on the cells. It is slower than the mouse test but provides a simple alternative to animal testing.[69,70] Other methods, including immunoassays, thin layer and high-performance chromatography, gas chromatography, mass spectroscopy, and nuclear magnetic resonance spectroscopy have been proposed and tested.[138]

54.4 CONCLUDING REMARKS

In recent years, there has been an increase in the incidence of human disease from fish and shellfish poisoning. This is well documented in the case of the US,[46] Europe,[21] Indo-Pacific,[25,67] and New Zealand.[65,66] The increase of disease has been associated with the appearance of two new shellfish poisonings—DSP and ASP—and one fish poisoning newly recognized to be important—Scombroid poisoning—the recognition of dozens of new species of toxigenic microalgae, and an apparent widespread dispersal or emergence of such algae in coastal waters around the world.[36,115,118] The relationship between human poisonings and the occurrence of toxigenic microalgae is clear, and most of the increase of disease incidence probably is due to the spread of these algae. However, other contributing factors are expanding tourism in tropical coastal areas and islands, growing imports of fish and shellfish from warm-water fisheries into North America and Europe, and improving recognition of seafood poisoning by physicians and other health professionals. Expanding tourism and growing imports have the same effect of exposing uninformed consumers to these products, while better diagnosis of these illnesses could inflate the statistics by revealing previously unrecognized cases. It should be noted that the population at risk from imports or tourism is no more susceptible to becoming ill from consumption of toxic fish or shellfish than the informed local inhabitant who eats such seafood since no immunity is conferred by previous experience; it is a matter of informed choice.

The reasons for the development of the prime cause of seafood poisoning increase are still in dispute. The spread of toxic dinoflagellates and other microalgae could be due, in part at least, to direct transport of the organisms by human action, such as in ship's ballast or incidental to direct transfer of aquatic species. In some situations, spread can be ascribed to changing ocean conditions due to naturally cyclic events (eg, El Niño phenomenon, global warming) or human alteration of habitat (eg, destruction of reefs) or pollution. The appearance of new toxigenic microorganisms is more difficult to understand. They may in fact simply have gone unobserved before because they were not actively looked for. There may be some credibility for this explanation in the case of DSP because the symptoms could be ascribed easily to an unknown bacterium, but given the extensive scale of outbreaks of DSP, particularly in Europe where scientists abound, this seems unlikely. Another explanation is that there has been an increase in toxicity within the incriminated toxigenic group, possibly by mutation. Identification of the microorganisms associated with toxification of fish and shellfish and mapping of their occurrence in world oceans is easily being actively pursued with some success, partly as a result of the application of methodologies derived from molecular biology including biochemical markers and RNA and DNA probes.[4,108] At least 57 species of toxic phytoplankton[118] and numerous species of toxigenic bacteria[29,127] have been reported.

The information derived from recent studies certainly has enhanced understanding of the origins and distribution of toxins through the marine ecosystem, but also has revealed that toxins rarely occur in a single chemical form and that they undergo change as they pass through the food chain.[12,15,62] This complicates the assay of toxicity in seafoods by chemical methods because different forms of a toxin show different potency. Another analytical problem now recognized is the concurrent presence of toxins from different species of toxigenic microorganisms in shellfish or fish: examples include PSP and DSP toxins in mussels,[72] palyotoxin and ciguatoxin in mackerel,[54] and possibly OA and ciguatoxin in "ciguateric" fish. Of course, the existence of such mixtures, if they really occur, raises problems for symptom definition of the disease in human patients.

The future pattern of occurrence of human seafood poisoning is difficult to predict. The ocean is a very large place (70% of the world surface is covered by water) and the current pace of discovery of toxic microalgae suggests there are more to be found; however, for shellfish poisoning at least there are grounds for hope. First, because there is a worldwide effort in coastal areas to identify toxic algae that successfully is outpacing human outbreaks, and second because countries increasingly are installing early warning procedures based on a combination of coastal phytoplankton surveys and toxin testing of edible shellfish. Under the best of circumstances, given such a system, the first large outbreak of shellfish poisoning should be the last because it would provide the necessary information to prevent future outbreaks.

Prevention of disease depends on intercepting toxic fish before they enter the food marketing system. This may require complete prohibition of landings of fish species with a high probability of toxicity or placing a burden of proof concerning nontoxicity on individuals or companies marketing such fish. Batch testing of fish is feasible for some toxins in particular situations, such as histamine testing of potentially scombrotoxic tuna and mahi mahi. Testing of individual fish already is enforced for puffer fish in Japan and the US, but may not be elsewhere in Southeast Asia. The problem is particularly difficult in the case of ciguatera because of the large number of species involved, the potential severity of the disease, and the widespread involvement of ciguateric species in subsistence fisheries. The latter is particularly difficult because fishermen have few other options to maintain their families, and it is well known that toxic levels can fluctuate widely in individual fish. In this case, as well as in more commercial situations, the need for an inexpensive test that can be applied at the point of capture is great.

It is encouraging that some progress has been made in the treatment of seafood poisonings, particularly with the apparent success of intravenous mannitol for ciguatera.[90] An unexpected but exciting development is the reported effectiveness of a monoclonal antibody for prophylaxis and treatment of tetrodotoxin poisoning in mice[98] because antigenic potential has been poor for seafood toxins in general. More research is needed into useful treatments to ameliorate the effects of fish and shellfish poisoning on the patient particularly because some of the toxins involved have been shown to be potentially carcinogenic.

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55 Long-Term Consequences of Foodborne Disease

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55.1 INTRODUCTION

It has been estimated that 5 to 35 of every 100 individuals in developed countries are affected by gastroenteritis each year and a large proportion of those infections are due to foodborne pathogens.[15] According to Archer and Young,[8] on the average, every United States (US) citizen suffers at least one episode of diarrhea annually. Most cases of diarrhea incurred by ingestion of a foodborne pathogen are mild, self-limiting, and of short duration. However, they should not be dismissed lightly because diarrheal episodes may have far-reaching effects that can be physically, psychologically, and economically catastrophic and may even result in death. The elderly and the young generally are more susceptible to foodborne diseases. In the US, the number of children less than 5 years of age has increased from 17.2 million in 1970 to 18.8 million in 1992, and the number of individuals more than 75 years of age has risen from 7.5 million to 13.8 million.[198] Projections in population growth show increases in the elderly population and in the number of children < 5 of approximately 100% and 15%, respectively, by the year 2025. Other developed countries probably will have similar statistics. Thus an ever-increasing, less immunocompetent population will be at risk for the consequences of foodborne diseases. In this chapter, we discuss some of the complications and long-term consequences that humans may experience from gastrointestinal insults due to foodborne pathogens.

55.2 REACTIVE ARTHRITIS AND REITER'S SYNDROME

55.2.1 The Diseases

The reactive arthritides (or seronegative spondyloarthropathies) include those arthritic syndromes characterized by sterile inflammation of the joints. These syndromes include reactive arthritis, Reiter's syndrome, and ankylosing spondylitis. The spondyloarthropathies characteristically involve the sacroiliac and peripheral joints and are seronegative for rheumatoid factor.[25] Other pathological features may include changes in the eyes with potential vision loss, the urinary tract, aortic valve, lung parenchyma, and skin, as well as changes at the sites of ligamentous insertion into bone. Importantly, the genetic makeup of certain individuals renders them more susceptible to the reactive arthritides.[25]

Gastrointestinal or genitourinary infections may trigger reactive arthritis or Reiter's syndrome.[3,214] Ebringer[44] has stated that ankylosing spondylitis follows *Klebsiella* infections, but Calin[25] and Russell and Almazor[146] disagree that there is a close relationship between arthritis and *Klebsiella* or other microorganisms. There is no compelling evidence that anklylosing spondylitis is a postenteric or postvenereal arthritis.

One to two weeks after an enteric or venereal infection, the patient with reactive arthritis presents with arthritis of several joints, generally those of the lower extremities—the knees and metatarsophalangeal (ball of the foot) joints. The arthritis usually is asymmetrical. Other symptoms may include back pain and inflammation at ligament-bone junctions (swelling of fingers and toes ["sausage digits"] and/or pain and tenderness beneath heels). The most common extra-articular manifestation in reactive arthritis is heaped-up scaling lesions present on the palms and soles (keratoderma blennorrhagica) and lesions on the buccal and pharyngeal mucosa, palate, and tongue. Conjunctivitis is present in approximately 30% of the patients, and a small number (< 5%) may have anterior uveitis (inflammation of iris, ciliary body, and choroid); cardiac involvement and urethritis may also occur. Ulcers of the glans penis (circinate balanitis) may be present in approximately 30% of patients with postvenereal reactive arthritis but are rare in patients with postenteric disease.[3,47,49]

Reiter's syndrome is a subtype of reactive arthritis and is characterized by a triad of symptoms: arthritis, conjunctivitis, and urethritis, which may occur together or sequentially. If one or more of these symptoms is absent, the term incomplete Reiter's syndrome is used.[3,47] Arthritis appears 2 to 6 weeks after the triggering infection; it is asymmetric and affects mainly the knees and ankles. Sausage digits, swelling at the insertion of the Achilles tendon, plantar fascitis (inflammation of the fibrous connective tissue in the sole of the foot), and low back pain are typical symptoms of Reiter's syndrome. Urethritis is manifested by dysuria and a thin, watery discharge; prostatitis and cystitis (inflammation of bladder) may be present. The conjunctivitis of Reiter's syndrome usually is mild; however, more severe complications such as iritis (inflammation of iris), uveitis, and corneal ulceration may develop. Other symptoms such as keratoderma blennorrhagica, ulcers of the glans penis, and oral lesions may be seen. Cardiac or neurological symptoms may be additional complications in some patients.[85,214] The clinical and pathological features of reactive arthritis and Reiter's syndrome often overlap.

The average duration of reactive arthritis or Reiter's syndrome is less than 6 months; however, some patients may show symptoms for more than 12 months.[85,100] For example, in a study of 25 patients with *Chlamydia*-induced reactive arthritis and 27 with arthritis triggered by enterobacteria, 10 of the *Chlamydia*-induced arthritis patients and 5 of those with enterobacteria-induced arthritis still showed clinical signs of arthritis after 1 year.[57] However, after 2 years, none of the *Chlamydia*-induced arthritic patients showed clinical symptoms and only one of the enterobacteria-induced arthritic patients was symptomatic. In a study of post-*Salmonella* reactive arthritis following a large food poisoning outbreak, Thomson et al.[190] found that chronic symptoms persisted for at least 5 years in a majority of the arthritic patients. The studies of Glennas et al.[57] and Thomson et al.[190] clearly indicate that the effects of foodborne enteritis may be long lasting in certain individuals.

Nonsteroidal anti-inflammatory drugs, intra-articular injections of steroids, and physical therapy are the treatments recommended for reactive arthritis. Antibiotic treatment in arthritis triggered by enteric pathogens does not appear to be effective in shortening the duration of the arthritis; however, use of antibiotics with chlamydial infections probably reduces the risk of postvenereal reactive arthritis.[100,175]

55.2.2 Role of HLA-B27 Antigen in the Diseases

There is a genetic predisposition for the reactive arthritides. Possession of the major histocompatibility complex (MHC) gene for the Class I antigen, HLA-B27, may predispose individuals infected with one of the triggering organisms to either reactive arthritis or Reiter's syndrome.[85] HLA antigens that cross react with HLA-B27, such as HLA-B7, also are associated with an increased risk for arthritis.[215] The frequency of the HLA-B27 allele in different populations varies; less than 1% of Japanese, 2% to 4% of American Blacks, and 6% to 10% of Caucasians are positive. The allele is absent in African Blacks and Australian aborigines.[30,127] Arthritis develops in approximately 2% of individuals (both HLA-B27 negative and positive) infected by a triggering organism, whereas approximately 20% of infected individuals that possess the HLA-B27 allotype will develop arthritis.[3] In a patient population suffering from reactive arthritides, 60% to 80% were HLA-B27+.[85]

Molecular mimicry generally is invoked to explain the association between possession of the HLA-B27 antigen and increased susceptibility to reactive arthritis. Infection might lead to formation of antibodies that cross react with region(s) of the HLA-B27 molecule having structural similarity to antigens of the triggering bacterium, thus resulting in autoimmunity.[54] Indeed, antisera from arthritic patients react with peptides from the HLA-B27 antigen;[54] however, the molecular mimicry theory does not explain the tissue specificity of the reactive arthritides. HLA-B27 is a Class I antigen of the MHC and is found in most somatic cells of the body, yet the pathology of the diseases is limited to a few tissues. In addition, bacteria not known to cause one of the reactive arthritides may also induce the generation of antibodies that cross react with peptides of the HLA-B27 molecule.[87] To further complicate matters, disease is not limited to HLA-B27+ individuals and most HLA-B27+ patients infected with a triggering organism do not develop arthritis. Thus, at present, it is unclear why HLA-B27+ individuals have an increased susceptibility to the reactive arthritides.

55.2.3 Microorganisms That Induce the Diseases

Microorganisms that trigger the reactive arthritides cause infections at mucosal surfaces, have lipopolysaccharides (LPS) on their outer membranes, and are intracellular pathogens.[112] The reactive arthritides are sterile arthritides that originate from infections at nonarticular sites. Immunohistological data of intestinal biopsy specimens of patients with reactive arthritides demonstrated the presence of *Yersinia enterocolitica* bacilli in mucosal, submucosal, and lymphoid tissue. Generally, *Y. enterocolitica* were not culturable in biopsied material.[36] Viable microorganisms are not isolated from the joints; however, microbial antigens are present.[112,193] A number of gastroenteritis-producing bacteria have been shown to induce the reactive arthritides. These include *Campylobacter jejuni*,[134] *Salmonella enteritidis*,[106,189] *S. typhimurium*,[76] *Shigella flexneri*,[60] *S. sonnei*,[96] *Yersinia enterocolitica*,[94] and *Y. pseudotuberculosis*.[99] Although *S. enteritidis* and *S. typhimurium* are the most common *Salmonella* serotypes that trigger arthritis, other serotypes such as *S. agona*, *S. montevideo*, and *S. saint paul* have been implicated.[111] The venereal pathogen, *Chlamydia trachomatis*, is the major cause of sexually acquired reactive arthritides.[85] Other organisms that have been implicated as triggers of reactive arthritides include *Borrelia burgdorferi*, *Chlamydia pneumoniae*, *Clostridium difficile*, *Giardia lamblia*, *Vibrio parahaemolyticus*, and *Ureaplasma*.[29,158,159,175,179] Vaccinations also appear to be capable of triggering reactive arthritis. A vaccine containing heat-killed *S. typhi* and a vaccine against hepatitis B virus have been linked to the induction of reactive arthritides.[67,112]

55.2.4 Microbial Determinants That May Be Involved in Induction of the Diseases

An important feature of reactive arthritides is that viable microorganisms are not present in the joints; however, bacterial antigens are detected. LPS from arthritis-causing *S. enteritidis*, *S. typhimurium*, *S. flexneri*, and *Y. enterocolitica* have been found in synovial cells (polymorphonuclear leukocytes and mononuclear phagocytes) of arthritic joints.[58-60] It is probable that after infection with the triggering organisms, bacterial LPS and other antigens are transferred from the site of infection to the joints via phagocytic cells.[55,193]

The pathology of the reactive arthritides is due to cell-mediated immune responses, ie, the diseases are T-cell mediated.[195] T cells isolated from the joints of reactive arthritis patients proliferated *in vitro* (incorporated radioisotope) to the greatest extent when they were exposed to the specific organism such as *Chlamydia*, *Campylobacter*, *Salmonella*, or *Yersinia* that triggered the arthritis.[56] A cationic 19-kDa antigen, a β-subunit of *Yersinia* urease, is a target antigen for synovial T cells isolated from patients with *Yersinia*-induced reactive arthritis and is arthritogenic in rats.[137,166] The cationic antigen may interact with negatively charged cartilage proteoglycan, which would allow the antigen to be retained in the joint as a persistent source of immune stimulus.[55,137] Other antigens that have been identified as targets of synovial T-cell response include a cationic 18-kDa chlamydial histone-like protein[55] and a 14-kDa 50s subunit of the ribosomal R2 protein from *Yersinia*.[195]

Heat shock proteins (HSPs) may be arthritogenic proteins targeted by synovial T cells.[55,154] The stress proteins are highly conserved with a great deal of amino acid homology in HSPs from different species.[136,154] Pathogens produce HSPs during invasion of a mammalian host, and these proteins serve to protect bacteria from host defense mechanisms.

Similarly, host cells synthesize homologous proteins in response to the stress of bacterial invasion. If bacterial HSPs become targets for T cells, it is possible for the host homologues to cross react immunologically, thus resulting in autoimmunity.[136,154] Hermann et al.[69] isolated a *Yersinia*-reactive T-cell clone from synovial fluid of a patient with Reiter's syndrome, which proliferated *in vitro* in the presence of *Yersinia* antigens and human 65-kDa HSP. However, Probst et al.[138] were not able to isolate T-cell clones from reactive arthritis patients that reacted with purified *Yersinia* 61-kDa HSP, the homologue of the human 65-kDa HSP used by Hermann et al.[69] At present, insufficient data are available to indicate whether cross-reactions between homologous HSPs from humans and bacteria play a role in the reactive arthritides.

Synovial T cells from patients with *Yersinia-*, *Salmonella-*, or *Chlamydia*-induced reactive arthritis show a limited spectrum of cytokine production; the major cytokines found were interferon-γ (IFN-γ) and interleukin-2 (IL-2). The cytokines IL-4, -5, and -10 were not produced.[68,93,151] Lahesmaa et al.[93] have suggested that the cytokines produced by helper T cells type 1 (Th1 cells) may be important in the generation and propagation of joint inflammation. Th1 cells produce IL-2 and IFN-γ and are involved in cell-mediated responses, such as delayed-type hypersensitivity. IFN-γ and IL-2 stimulate the activity of cytotoxic T-cells, macrophages, and natural killer cells.[132] The IL-4, -5, and -10 pattern of cytokine production is found in Th2 cells. The Th2 cell cytokines are involved in humoral-mediated immunity and assist in antibody production by inducing B-cell differentiation and expansion. The cytokines are also involved in promoting allergic type responses.[132] Cross-regulation of the subsets occurs because IFN-γ can inhibit cytokine production by Th2 cells. IL-4 and IL-10, produced by Th2 cells, can suppress (down-regulate) Th1 responses.[132]

While Lahesmaa et al.[93] proposed that the cytokine products of Th1 cells play a role in the pathology of reactive arthritis, Schlaak et al.[151] demonstrated that Th1 cells actually may prevent arthritis. Th1 cells control the intracellular replication of *Yersinia enterocolitica* in phagocytic cells. IFN-γ promoted intracellular killing of *Yersinia* by activating cytotoxic cells whereas IL-4 (produced by Th2 cells) antagonized IFN-γ activation. In addition, the Th2 cytokine, IL-10, inhibited the *in vitro* proliferation of *Yersinia*-specific T-cells and down-regulated production of IFN-γ and IL-2.[151] Thus, the data of Schlaak et al.[151] indicated that Th1 cell production of IFN-γ promotes the destruction and elimination of intracellular *Y. enterocolitica*. Therefore, it is probable that the pathology of the reactive arthritides is not due to excessive Th1 cell expansion *per se* but rather is due to an imbalance of Th1 and Th2 cell activities.

Arthritogenic pathogens may produce proteins that behave as superantigens and stimulate T-cells. Stuart and Woodward[174] have demonstrated an antigen in *Y. enterocolitica* that behaved as a T-cell superantigen and a superantigen from *Y. pseudotuberculosis* has been isolated, purified, and partially sequenced.[2,197,213] Nothing is known concerning superantigen activity in other microorganisms that induce reactive arthritides. Superantigens can stimulate proliferation of a large number of T cells bearing a particular variable region of the β chain (Vβ sequence) of the T-cell receptor. Each superantigen will recognize one to five different Vβ sequences. As many as 5% to 25% of T cells having the proper Vβ sequences may be activated by a superantigen, whereas a conventional antigen may engage only about 0.01% of the T-cell pool. The result of such massive T-cell stimulation is substantial cytokine release, including IL-2, -4, and -6, tumor necrosis factors, and IFN-γ.[75,152,164,183,222]

Lahesmaa et al.[92] visualized the role of bacterial superantigens in the reactive arthritides in the following manner: inflammation of the joint may result from massive cytokine production due to superantigen stimulation of large numbers of T cells. Alternatively, the superantigen could contribute to inflammatory synovitis by expanding a population of autoreactive T cells that recognize a particular synovial antigen, but a role of superantigens in the pathology of the reactive arthritides is purely speculative at the present time.

Bacterial LPS are present in synovial fluid cells,[58-60] but are not considered arthritogenic antigens because LPS do not activate T cells.[68] The outer-membrane proteins (OMPs), YadA and YopH, of *Y. enterocolitica* may contribute to arthritogenicity in rats because mutant strains lacking the ability to produce these OMPs demonstrated decreased arthritis-inducing ability.[53,63] YadA binds to extracellular matrix proteins such as collagen, laminin, and fibronectin present in joints.[48,62,155,156,178,185] A *Y. enterocolitica* strain lacking the chromosomal gene that encodes the iron-chelator, yersiniabactin, did not induce arthritis in rats.[53] It is not known whether the *Y. enterocolitica* OMPs or yersiniabactin play a role in the induction of the reactive arthritides in humans.

It appears that the response of synovial T cells to antigens of the arthritis-triggering microorganism plays an important part in the pathogenesis of the reactive arthritides; however, it is unclear which bacterial antigen or antigens are arthritogenic and activate T cells. Recent reviews have discussed the relationship between foodborne pathogens and the reactive arthritides[167,169] and the immunology of these disorders.[23]

55.3 GUILLAIN-BARRÉ SYNDROME

55.3.1 The Disease

The Guillain-Barré syndrome (GBS; acute inflammatory polyneuropathy) is a demyelinating disease of the peripheral nerves. Since the suppression of polio, GBS is now the most common cause of paralysis in developed countries.[143] In the US, the annual incidence of the disease is 1 to 2 cases per 100,000 people. Approximately two thirds of the cases follow an antecedent event such as bacterial or viral infection, surgery, immunization, or drug treatment.[143] Unlike the reactive arthritides, GBS does not have a familial association.[170]

Acute GBS begins with abnormal sensations and loss of touch in toes and fingers followed by difficulty walking and climbing stairs due to leg weakness. Weakness ascends from lower limbs to involve the trunk, upper limbs, and cranial nerves. Pain may be present in neck, shoulder, and back regions. Some patients have weakness of the facial and oropharyngeal muscles, and approximately 25% of patients have involvement of the respiratory muscles; a few of these patients may need assisted ventilation. There may be dysfunction in other organs enervated by the autonomic nerves (such as the heart and gastrointestinal and urinary tracts). The brain and spinal cord are not affected. Due to general paralysis, the patient usually is bedridden.[119,133,139,143,170,221] Within 3 to 12 months, most patients recover completely or are left with only minor abnormalities that do not interfere with a normal lifestyle; however, 10% to 20% of GBS patients have significant residual weakness that prevents resumption of prior occupation or lifestyle. Death occurs in 1.5% to 8.0% of the patients.[119,139,143,170] Death may be due to nosocomial (hospital-acquired) infections, respiratory distress syndrome, pulmonary emboli (blood clots), or cardiovascular complications.

In GBS, inflammatory lesions are scattered throughout the peripheral nervous system but are not present in the central nervous system. These lesions are characterized by invasion of the myelin sheath by macrophages, which strip myelin from intact axons. Axonal loss may also occur.[119,143,188] Both humoral and cellular immune mechanisms appear to be involved in GBS. Antiperipheral-nerve myelin antibodies are found in GBS patients and are highest when neurological symptoms appear; declining antibody titers against peripheral-nerve myelin correlate with clinical improvement.[90,206] These antiperipheral-nerve myelin antibodies exert their demyelinating action through complement activation. In addition, at least one fourth of GBS patients exhibit high levels of antibodies to the GM_1, GM_2, and GD_{1b} gangliosides.[211] Complement fixation and inflammation of neural tissue by antiganglioside antibodies may induce the pathology seen in GBS patients.

T-cell responses to peripheral nerve proteins are also involved in the pathogenesis of GBS.[86] There is proliferation of T cells and increased levels of soluble interleukin-2 receptors (sIL-2 R) in GBS. Increased serum concentrations of sIL-2 R indicate the presence of circulating activated T cells, which may be relevant to GBS pathogenesis.[65] Activated T cells may assist B cells in the production of antibodies against myelin components and may regulate, recruit, and activate macrophages that attack the myelin sheath.[33,65] In a later study, Hartung et al.[66] actually demonstrated that the cytokine interleukin-2 was markedly increased in GBS patients, and as patients recovered from the disease the level of the cytokine returned to normal, suggesting that interleukin-2 has a pathogenic role in GBS. The concentration of the cytokine, tumor necrosis factor-α, was increased significantly in approximately 25% of GBS patients, suggesting that this cytokine also may play a role in the pathogenesis of the disease.[46] Honavar et al.[71] suggested that the T-cell response may be the major cause of demyelination in some GBS patients, whereas antibody-mediated demyelination may be more important in other patients. T-cell and B-cell responses generally act in concert, and the different pathologies seen in GBS probably reflect the relative contribution of each cell type.[71]

Plasmapheresis (plasma exchange) or administration of intravenous immune globulin, if used early, can alleviate the effects of GBS.[40,191,200,201] The benefits of plasmapheresis probably result from the removal of harmful antibodies such as demyelinating antibodies.[191] It is not clear why intravenous immunoglobulin is effective. The use of plasmapheresis requires sophisticated equipment and trained personnel, whereas intravenous immunoglobulin treatment is less complicated. Although plasmapheresis and intravenous immunoglobulin aid in the recovery of GBS patients, supportive care and rehabilitation of the patient are of primary importance.[39] Recovery is slow, requiring several weeks to months. Many GBS patients require extended and scrupulous intensive care including positive-pressure ventilation in cases with respiratory failure, nutritional support via nasogastric tube feeding, prevention of pressure on peripheral nerves, and prevention of nosocomial infections. Most patients need physical and occupational therapy as well as strong psychological support.[144,170]

55.3.2 Variants of Guillain-Barré Syndrome

Miller-Fisher syndrome (MFS) is an acute, self-limiting neuropathic disorder characterized by ophthalmoplegia (paralysis of eye muscle), ataxia (muscle incoordination), arcflexia (absence of reflexes), and facial weakness. This variant accounts for approximately 5% of GBS cases and, except for ophthalmoplegia, the clinical features overlap with GBS.[141] Antibodies to GQ_{1b} ganglioside are present in most cases of MFS.[212,218] Roberts et al.[141] presented evidence suggesting that a factor in sera of MFS patients, probably the anti-GQ_{1b} antibody, is responsible for muscle weakness. As in the case of GBS, plasmapheresis or intravenous serum globulin treatment aids in recovery of patients with MFS.[171,217]

Chinese paralytic syndrome (CPS; also known as acute motor axonal neuropathy) is easily confused with GBS; CPS differs from GBS in that the disease has a summer seasonality, usually involves children, and occurs predominantly in rural areas of northern China.[117,118] CPS cannot be distinguished from GBS clinically but the disorders are distinguishable by electrodiagnosis. In CPS, there is little evidence of demyelination; the disorder may result from a lesion in a reversible distal motor nerve terminal or anterior horn cell.[117,118] As in GBS, high titers of IgG anti-GM_1 are present in patients with CPS.[89] Cases similar to CPS have been reported elsewhere in the world, and the disease is probably not limited to China.[117]

55.3.3 Microorganisms That Induce the Disease

There have been a few reports of infection with *Borrelia burgdorferi*, *Mycoplasma pneumoniae*, and *Salmonella typhi* before the appearance of GBS,[64,73,147] but infections by *Campylobacter jejuni* are the major bacterial cause of GBS.[120,139] In the US, 20% to 40% of GBS cases show evidence of recent *C. jejuni* infection. The annual incidence of *C. jejuni* infection prior to the appearance of GBS has been estimated to range from 0.17 to 0.51 cases per 100,000 people.[120] MFS and CPS also have been associated with *C. jejuni* infections.[21,216]

55.3.4 Microbial Determinants That May Be Involved in Induction of the Disease

GBS may be induced by infection with *C. jejuni* if an immune cross-reaction between peripheral nerve antigens and bacterial antigens occurs. Antibodies to neural gangliosides have been found in sera from 231 of 847 (27.3%) GBS patients; the most common antibody is directed against the ganglioside, GM_1.[211] GBS patients with antibodies that react with GB_1 or GD_{1b} gangliosides also show a higher incidence of prior *Campylobacter* infections than other GBS patients.[204,209] *C. jejuni* LPS isolated from strains associated with GBS have regions homologous to the human gangliosides GM_1 and GD_{1b}.[12,13,219] Patients with MFS show high antibody levels against the ganglioside GQ_{1b}.[212,217] Yuki et al.[220] demonstrated that the GQ_{1b} epitope was present on LPS from *C. jejuni* strains isolated from patients with MFS. Molecular mimicry between homologous regions of the bacterial LPS and the peripheral-nerve gangliosides may lead to autoimmunity and GBS or MFS.

Campylobacter jejuni toxin binds to GM_1; this toxin-GM_1 complex could alter recognition of self antigens and thus trigger the production of antibodies directed against neural antigens.[139] There are no data, however, supporting the role of the toxin in induction of GBS. Fujimoto and Amako[52] found that antisera from patients with *C. jejuni*-induced GBS reacted with the myelin proteins P0 and P2. Antibacterial antibodies that cross react with myelin proteins may lead to an autoimmune reaction with resultant GBS. Rees et al.[139] suggested that *C. jejuni* may induce GBS via superantigens. The superantigen could activate large numbers of antimyelin or antineural T cells resulting in the production of high levels of cytokines, which could damage nerve tissue and induce GBS; however, there is no evidence that *Campylobacter* species synthesize superantigens.

There is evidence, therefore, of homology between components of *C. jejuni* and antigens in peripheral-nerve gangliosides and in myelin proteins. It is possible that immune reactions against *C. jejuni* antigens may induce GBS and MFS. Smith[168] has reviewed the role of *C. jejuni* in the induction of GBS and related diseases.

55.4 HEMOLYTIC UREMIC SYNDROME

55.4.1 The Disease

Hemolytic uremic syndrome (HUS) is a triad of microangiopathic hemolytic anemia (disease in which small blood vessels become distorted, resulting in damage or destruction of red blood cells), thrombocytopenia (abnormally small number of platelets in the circulatory system), and acute renal failure. The incidence of HUS is highest in early childhood but may appear in all age groups; the syndrome is the major cause of renal failure in children in the US.[10] Thrombotic thrombocytopenic purpura (TTP) is similar to HUS in histopathology and clinical aspects; however, the syndrome usually occurs in adults, appears to affect women more than men, is rare in children and infants, and prominent signs include fever and central nervous system involvement. It is not certain whether TTP and HUS are separate entities or merely represent different clinical endpoints of a common pathological process.[10] Remuzzi et al.[140] suggested that HUS and TTP are different clinical expressions of the same disease; renal failure is the prominent feature in children (HUS), whereas neurological involvement is the main feature in adults (TTP). However, Neild[124] stated that approximately 50% of patients with diarrhea-associated HUS have fever and that neurological involvement is common; thus, the distinguishing differences between HUS and TTP may be blurred.

Typical HUS is diarrhea-associated, with the enteric pathogen *Escherichia coli* O157:H7 as the main identified cause in the US, while atypical HUS is not preceded by nor associated with diarrhea. Atypical HUS has been associated with pregnancy, transplantation, malignancy, and use of certain drugs such as cyclosporin A, mitomycin C (or other immunosuppressive drugs), antibiotics, or oral contraceptives.[81]

Endothelial cells are the primary sites of injury in HUS.[121] The main target organ is the kidney, but other tissues such as the gastrointestinal tract (small and large intestine, pancreas, and liver), cardiovascular system, and the central nervous system may also be affected.[124,162] The organ or organs affected show the same underlying pathology, ie, thrombosis of the microcirculatory system due to platelet adhesion and aggregation at the sites of endothelial injury.[124]

In symptomatic, Shiga-like toxin (SLT)-producing *E. coli* O157:H7 infections, there is a prodromal illness of crampy abdominal pain and self-limited fever. Within 1 to 2 days, nonbloody diarrhea develops and progresses to bloody diarrhea in about 90% of US cases.[182] Vomiting may occur at any time during the illness. Approximately 7 days after the initiation of diarrhea, 5% to 10% of the patients less than 10 years of age present with HUS. The syndrome probably results from vascular endothelial damage in renal tissue following absorption of SLT from the infected gut.[181,182] Narrowing of renal arterioles and glomerular capillaries due to endothelial cell swelling and deposition of fibrin and platelet aggregates lead to mechanical damage or fragmentation of red blood cells and platelets as they traverse occluded microvessels.[82,121,145] Because all of the circulating blood of the body passes through the kidneys every 4 to 5 minutes,[79] it is not surprising that the resulting red cell damage leads to anemia. Adherence of platelets to the injured capillary walls contributes to the formation of microthrombi with further narrowing of the lumen of the vessels and leads to thrombocytopenia. Occlusion of the capillaries of the glomeruli results in reduced glomerular filtration; complete blockage of the renal microvessels can lead to tubular and glomerular necrosis. Thus, the overall effect of HUS is renal dysfunction.[82,121] Blockage of the microcirculation of other organs of the body may occur also in HUS.[124,162]

In TTP, the mortality rate can be greater than 90% in untreated patients; however, the majority (up to 85%) of children with HUS recover spontaneously or need only supportive treatment.[35,123] In many cases, treatment may be limited to rehydration of the patient and psychological reassurance of both child and parents. Tarr[182] recommends no antibiotic treatment for *E. coli* O157:H7 infections because antibiotics may lyse intracolonic pathogens, causing release of SLTs. In addition, Tarr[182] does not advocate the use of antimotility agents or narcotics in *E. coli* O157:H7 infections. Although these medications alleviate intestinal pain, they delay clearance of the pathogen from the gut and permit further SLT production. In mild cases of diarrhea-induced HUS without anuria, Kaplan[80] recommended rehydration if required, maintenance of hemoglobin level (by red blood cell transfusions), control of hypertension, and psychological support. If anuria is present, dialysis in addition to the other supportive measures is recommended.[80] In some HUS patients, there may be gastrointestinal, cardiovascular, or neurological involvement in addition to renal involvement and appropriate corrective measures must be initiated on a case-by-case basis.[107,162] In some patients, death may occur or there may be long-term physiological and neurological deficits.[107,162]

Intensive plasma infusion with fresh frozen, platelet-poor plasma was recommended by Kaplan[80] as a treatment for TTP patients; however, Daniel[35] indicated that plasma exchange (with fresh frozen plasma as the replacement fluid) may be more efficacious than plasma infusion in TTP. It is not clear whether the beneficial results of plasma infusion or exchange are due to removal of harmful agents (exchange) or replacement of essential entities that have become depleted (both exchange and infusion).[35]

55.4.2 Role of Blood Group and HLA Antigens

Taylor et al.[184] suggested that the P blood group may play a role in diarrheic HUS because SLTs can bind to ceramide groups on the P blood group glycolipid antigens. Red blood cells (RBCs) from 80% to 95% of the normal population have the P1 blood group phenotype (comprising the P, P_1, and P^k antigens) and most of the remainder of the population have the P2 phenotype (P and P^k antigens but lacking the P_1 antigen). SLT-I and SLT-II bind to glycolipids containing terminal Gal-α(1→4)-Gal residues. The P^k antigen, which is Gb_3, globotriaosylceramide, and the P_1 antigen contain terminal Gal-α(1→4)-Gal residues. SLTs bound to RBCs with the P1 and P2 phenotypes but did not bind to RBCs of the p (P^{k-}, P^-, and P_1^-) phenotype.[20] SLTs showed reduced binding of RBCs deficient in the P^k antigen. There was a 10-fold decrease in binding of P2 phenotype RBCs by SLT-I and SLT-II compared with binding to P1 phenotype cells.[20] It was suggested that binding of SLTs to P1 phenotype RBCs is enhanced due to the presence of Gal-α(1→4)-Gal residues on both the P^k and P_1 antigens and that the P^k antigen may be less accessible to SLT binding on P2 phenotype RBCs than on P1 phenotype RBCs. Red blood cells do not synthesize protein and are resistant to the action of SLT. Taylor et al.[184] postulated that, by binding to red blood cells containing the P_1 antigen, SLT is rendered less available for toxic activity on nucleated endothelial cells.

Newburg et al.[126] and Taylor et al.[184] presented preliminary evidence indicating that the proportion of HUS patients with the P_1 antigen was significantly less than that of control patients and that the lack of P_1 antigen was more common in patients with severe HUS. These results suggest that the absence of the P_1 antigen is a risk factor for HUS; however, Robson et al.[142] found that HUS patients were as likely to have the P_1 antigen as were control patients. In these three studies,[126,142,184] the patient population was small, and it appears premature to draw any definitive conclusions concerning the role of P_1 blood group antigen in HUS.

Diarrheic HUS appears to show a greater incidence in children harboring the Class I HLA-B40 antigens.[160,199] These include HLA-B40, its splits (the closely related HLA-B60, -61, -41, -47), and the cross-reactive antigens -B44 and -B13. The number of patients studied was small (n = 89) and to evaluate properly the role of HLA-B40 antigens as risk factors for HUS, HLA typing of a large number of patients must be performed.

55.4.3 Microorganisms That Induce the Disease

Typical (epidemic or diarrhea-associated) HUS is a result of an infection with Shiga toxin-producing *Shigella dysenteriae* type 1 or SLT-producing enterohemorrhagic *E. coli* (EHEC). There is generally a prodrome of bloody diarrhea with *S. dysenteriae* or *E. coli* infections prior to HUS.[81] Reports from various parts of the world indicate that the most common cause of diarrhea-associated HUS is *E. coli* O157:H7. Marks and Roberts[114] have estimated that in the US, 3 to 8 individuals per 100,000 population acquire an *E. coli* O157:H7 infection each year. Five to ten percent of those infected individuals less than 10 years of age will develop HUS[125] and 4% to 13% of patients with diarrhea-associated HUS do not survive.[140] A recent estimate from the US Centers for Disease Control and Prevention indicated that there is an annual minimum of 20,000 cases of *E. coli* O157:H7 infection and 250 deaths.[26]

Other SLT-producing *E. coli* serotypes have also been implicated in HUS.[61,83] *E. coli* serotype O103:H2 was isolated from stools of 6 of 69 children with HUS in France between 1987 and 1989.[113] By colony blot hybridization using labeled DNA probes, Cordovéz et al.[31] found *E. coli* serogroups O157, O26, and O111 in the stools of children with HUS. All of the isolates hybridized with an EHEC plasmid probe and with either an SLT-I probe or probes for SLT-I and SLT-II. Most *E. coli* O157:H7 strains do not ferment sorbitol within 24 hours; therefore, differential media containing sorbitol are frequently used in clinical settings to screen bloody stools for the presence of the organism. However, looking only for *E. coli* O157:H7 using these media may lead to an underestimation of the number of cases of HUS caused by other SLT-producing serotypes.

More rarely, HUS may be a result of infection by neuraminidase-producing *Streptococcus pneumoniae*.[81,107] Other microorganisms, bacterial and viral, have been implicated in the causation of HUS but the reports concerning these organisms are anecdotal and their role in HUS is unsubstantiated.[81] However, one report described SLT-II-producing *Citrobacter freundii* as the causative agent of a nursery school outbreak of severe gastroenteritis and HUS in a small town in northwest Germany.[196]

The primary requirement for an agent to induce HUS is the ability to damage endothelial cells of the vascular system. Shiga toxin, SLT, neuraminidase, LPS, phospholipase C, mitomycin C, and cyclosporin A each have been shown to injure vascular endothelial cells.[81]

The incidence of TTP is approximately one per million population and therefore it is uncommon in comparison with HUS.[80] Cases of TTP resulting from infections by SLT-producing *E. coli* associated with a prodrome of bloody diarrhea have been reported;[10] however, Kaplan[80] stated that patients with "TTP" induced by SLT-producing *E. coli* probably had HUS rather than TTP. In Kaplan's view,[80] a definitive cause for TTP has not been established.

55.4.4 Microbial Determinants That May Be Involved in Induction of the Disease

Adherence and colonization are necessary first steps in induction of disease. One of the putative attachment factors for *E. coli* O157:H7 induces the attaching and effacing (A/E) cytoskeletal lesion. The histopathology of A/E is characterized by intimate bacterial attachment to intestinal epithelial cells, effacement of microvilli, rearrangement of the cellular cytoskeleton, and cupping of the cell membrane around the bacterium in a pedestal-like formation.[78] Microfilamentous actin (F-actin) accumulates beneath the cytoplasmic membrane of the host cells at sites where bacteria have attached. The *eaeA* gene necessary for expression of A/E in *E. coli* O157:H7 has been cloned and sequenced.[17] This gene is 97% homologous to the enteropathogenic *E. coli eaeA* gene over the first 2,200 base pairs (bp), and there is only 59% homology over the last 800 bp. Some *Citrobacter freundii* and *Hafnia alvei* strains also produce A/E lesions and possess DNA homologous to the *E. coli eaeA* gene.[51,150] There are also similarities between the sequence of the *eaeA* gene and that of the *Yersinia inv* (invasin) gene. The gene product of the enteropathogenic *E. coli eaeA* is a 94-kDa outer membrane protein, intimin, which is necessary but not sufficient to produce A/E lesions.[38] The EHEC *eaeA* gene protein product is 97 kDa and is termed intimin$_{O157}$.[109] The majority of SLT-producing *E. coli* strains (O157:H7 and other serotypes) causing disease in humans and cattle possess an *eae* gene.[108]

Other determinants as well as *eaeA* appear necessary for production of A/E.[42] A 35-kbp genetic locus designated LEE was found to consist of multiple regions involved in the formation of A/E lesions.[115] This locus is conserved among enteropathogenic *E. coli*, EHEC, and other organisms that have been shown to produce A/E lesions, such as *Hafnia alvei* and *Citrobacter freundii*, but was absent in nonpathogenic *E. coli* and in other non-A/E bacteria. The LEE region contains *eaeA*, *eaeB*, *sepA*, and *sepB* loci that encode determinants necessary for A/E lesion formation. The *selC* (selenocysteine) tRNA locus was shown to be the LEE insertion site on the *E. coli* chromosome.[115]

EHEC are not invasive when tested by the Sereny test;[186] nonetheless, strains of *E. coli* O157:H7 can invade certain human cell lines.[131] Invasion ability is encoded chromosomally and depends on short-lived invasion protein(s) produced by the bacteria and also on the presence of eukaryotic microfilaments. Use of cytochalasin D, a potent depolymerizer of microfilaments, inhibited internalization of the bacteria; however, invasion of enterocytes may not be necessary in induction of disease because the organism produces large amounts of SLT, which results in rapid cell death.[186]

Iron is necessary for the growth of microorganisms *in vitro* and *in vivo*; thus, iron-acquiring mechanisms can be considered as virulence factors in pathogens. Various serotypes of EHEC produce temperate bacteriophage or plasmid-coded hemolysins.[19,153] Beutin et al.[19] suggested that there may be a close relationship between enterohemolysins and SLT production because they found that 89% of EHEC strains produce both factors. Law and Kelly[97] found that 20 of 20 strains of SLT-producing O157 strains produced enterohemolysins and growth of all O157 strains was stimulated by the hemoglobin and heme released by action of the enzyme. However, only 22 of 33 SLT-producing non-O157 strains produced enterohemolysins; the growth of 7 of 33 strains was stimulated by heme, whereas growth of only 4 of 33 was stimulated by hemoglobin. Law and Kelly[97] suggested that the production of enterohemolysins and ability to use products of hemolysis may account for the greater incidence of bloody diarrhea and HUS seen with *E. coli* O157 than with non-O157 strains.

Iron in excess of that needed for bacterial growth depresses synthesis of Shiga toxin and SLT-I but not SLT-II or SLT-IIv (currently designated SLT-IIc).[104] The chromosomal gene *fur* regulates the temperate bacteriophage *slt* operon (genes necessary for expression of SLT). The *fur* gene product, the Fur protein, acts as a corepressor with Fe^{++}; the Fur-iron complex binds to the Fur-binding site of the *slt* gene promotor and prevents the transcription of SLT-I.[104] SLT-II and SLT-IIc genes do not contain sequences in their promotor regions for binding of the Fur repressor protein.

The toxins of the Shiga family appear to be the most important virulence determinants in hemorrhagic colitis and HUS.[163] The genes for Shiga toxin are located on the chromosome, whereas those for SLT-I and SLT-II are encoded by toxin-converting bacteriophages. The amino acid and DNA sequences of Shiga toxin and SLT-I are similar with greater than 99% genetic homology. The two toxins have similar biologic activities and cross-react immunologically.[172] SLT-I and SLT-II are different antigenically; however, they show cytotoxicity for the same cell lines and possess similar lethal and enterotoxic activities.[173] Amino acid and DNA sequence homologies between SLT-I and SLT-II are 56% and 58%, respectively.[77] Shiga toxin and the SLTs are subunit toxins consisting of an "active" A subunit and multiple "binding" B subunits. The B subunits bind to the receptor (Gb_3) on the mammalian cell surface; following internalization, the A subunit is cleaved into the A1 and A2 fragments. The A1 fragment disrupts protein synthesis by removing an adenine residue from the 28S rRNA component of the 60S ribosomal subunit and thereby blocks peptide elongation. Shiga toxin, SLT-I, and SLT-II block protein synthesis by the same mechanism.[70,83,128]

Human umbilical vascular endothelial cells (HUVEC) and human renal microvascular endothelial cells (HRMEC) are damaged by low levels of Shiga toxin.[129,130] Cell viability and protein synthesis of renal endothelial cells were reduced by 50% with Shiga toxin at levels of 1 pM, whereas more than 1 nM of Shiga toxin had no effect on umbilical endothelial cells. The Gb_3 level of renal endothelial cells was approximately 50 times that of umbilical cells.[130] Gb_3 is a major component of renal tissue; the Gb_3 level was higher in the renal cortex than in the medulla, which correlates with the location of lesions seen in HUS.[22] However, the Gb_3 level in infant kidney was considerably less than that in adult kidney, which does not correlate with the apparent increased susceptibility of children to HUS. Lingwood[103] studied the binding of fluorescein-conjugated SLT-1 to frozen human renal sections and found that SLT was detected in infant (< 2 years of age) glomeruli but not in adult. SLT-binding by infant glomeruli correlated with the observed incidence of diarrheic HUS in children. It appears that concentration of Gb_3 in renal tissue may not be the critical factor in HUS.

Impairment in the normal function of the renal fibrinolysis system may contribute to the pathology of HUS.[110] HUVEC and HRMEC differ in types and levels of fibrinolysis factors produced such as tissue-type and urokinase-type plasminogen activators and plasminogen activator inhibitor-1 (PAI-1). HUVEC are antifibrinolytic because expression of PAI-1, the prime inhibitor of fibrinolysis factors, is high, whereas HRMEC produce low amounts of PAI-1, and therefore are profibrinolytic. Incubation of the cells with Shiga toxin resulted in a greater reduction in total cellular protein synthesis and cell viability for HRMEC than for HUVEC, and the production of fibrinolysis factors was decreased to a greater extent than total protein synthesis in HRMEC.[110] Thus Shiga and Shiga-like toxins may effect a decrease in renal fibrinolytic activity, leading to thrombosis.

The role of the Shiga family of toxins in disease may be visualized as follows: the A1 fragment of the toxin disrupts protein synthesis by the endothelial cells of the intestinal vasculature leading to cell death and hemorrhagic colitis. With the breakdown of intestinal integrity, toxin enters the circulatory system and can locate in the kidneys and other organs and cause damage to vascular endothelial cells. Endothelial cell death in the renal vasculature (or vasculature of other organs) allows circulating blood to be exposed to the underlying collagen with initiation of coagulation. The resultant narrowing of the vessel lumens eventually leads to HUS or complications in other organs.[70,81,135]

A satisfactory explanation of the mechanism of SLT induction of HUS is hampered by the lack of suitable animal models. Animal models have been described, including a mouse model,[102,207,208] a gnotobiotic pig model,[41] and a rabbit model;[165] however, none of the models emulate completely the disease syndrome seen in human EHEC infections. Tarr[180] stated that the rabbit model of Sjogren et al.[165] holds promise as a small-animal model that may provide much-needed answers to the involvement of SLTs in HUS.

55.5 OTHER COMPLICATIONS AND LONG-TERM CONSEQUENCES OF FOODBORNE DISEASE

Immunologic phenomena involving autoimmune reactions with production of immune complexes or of antibodies against "self" antigens and immunologic responses to bacterial antigens and superantigens may result in complications following infection with enteric pathogens. Several of these complications are discussed.

55.5.1 Glomerulonephritis

Glomerulonephritis (acute/postinfectious) may develop after an infection with group A nephritogenic streptococcal strains (poststreptococcal glomerulonephritis) or following infections with other agents, including enteric pathogens. Postinfectious glomerulonephritis is usually associated with deposition of immune complexes within the glomerulus activating a cascade of events leading to tissue destruction. *Campylobacter jejuni* antigen was identified in the glomeruli of a 5-year-old child who had suffered from *C. jejuni* enteritis with bloody diarrhea several weeks earlier.[7] A *Shigella* virulence protein antigen was found in immune complexes isolated from the serum of a patient who had dysentery due to *S. flexneri* type 1B and who developed acute glomerulonephritis.[122] *Streptococcus zooepidemicus*, an infrequent human pathogen, was associated with a milkborne outbreak of infection complicated with glomerulonephritis.[50] Staphylococcal superantigens may also be involved in the induction of nephritis by triggering the production of high levels of cytokines, formation of circulating immune complexes, and glomerular deposition of IgG, IgA, and complement components.[91]

55.5.2 Erythema Nodosum

Erythema nodosum is an immune-mediated dermatologic complication characterized by painful nodular lesions that change from pink to bluish to brown and are commonly found on the legs and arms. *Campylobacter*, *Yersinia*, and *Salmonella* infections have been associated with the occurrence of erythema nodosum.[4,43,45,95,148,161] The lesions may be recurrent with prolonged fecal excretion of pathogens such as *C. jejuni*.[11]

55.5.3 Kawasaki Syndrome

The etiology of Kawasaki syndrome (KS; also known as mucocutaneous lymph node syndrome or Kawasaki disease) is unknown; however, the clinical features and laboratory findings strongly suggest that KS may be induced by infectious organisms.[24] The syndrome is characterized by vasculitis of large- and medium-sized arteries, in particular, the coronary vessels, fever, polymorphous rash, conjunctivitis, pharyngitis, strawberry tongue, red fissured lips, erythema and edema of the palms and soles, and cervical lymphadenopathy. KS usually affects infants and children less than 5 years of age; in the US and Japan, KS is the most common cause of acquired heart disease in young children.[24] Immunologic features of KS include a marked expansion of T cells possessing the Vβ2 and Vβ8 receptors, increased production of cytokines including tumor necrosis factors and interleukins, and complement activation.[1,88] The powerful immune response that occurs with KS leads to destruction of arterial walls.

Toxic shock syndrome caused by *Staphylococcus aureus* toxic shock syndrome toxin-1 (TSST-1) and streptococcal pyrogenic exotoxins, classified as superantigens, shares many clinical and immunologic features with KS. Superantigen-producing *Staphylococcus* and *Streptococcus* strains may be involved in the induction of KS.[6,32,101,192] Leung et al.[101] isolated TSST-1-producing *S. aureus* and streptococci producing pyrogenic exotoxins B and C from 13 of 16 KS patients, but from only 1 of 15 control patients. The strain isolated from the control patient typically was gold colored, whereas the strains isolated from KS patients were white and produced only low levels or no hemolysin.

55.5.4 Crohn's Disease and Ulcerative Colitis

Both Crohn's disease (CD) and ulcerative colitis (UC) are characterized by chronic inflammation of the gastrointestinal tract. Although there are differences in the pathologic and clinical features of CD and UC, they are classified collectively as idiopathic inflammatory bowel disease (IBD). Because CD and UC resemble gastrointestinal infections caused by infectious microorganisms, and clustering of cases of CD among family members[202] and close contacts[5] have been reported, an infectious etiology has long been suspected. Furthermore, there is evidence that enteric infections are associated with exacerbations and relapses of established IBD.[149] Erythema nodosum, eye abnormalities, arthritis, and other symptoms can occur as extraintestinal manifestations of CD.

One pathogen under investigation as a possible cause of CD is *Mycobacterium paratuberculosis.* Because CD and Johne's disease (JD), which occurs in ruminants and other animals including primates, share similar clinical and pathological features and *M. paratuberculosis* is the causative agent of JD, it has been suggested that the disorders share a common etiology. Cows excrete *M. paratuberculosis* in their milk and colostrum,[176] and the organism apparently is more resistant to pasteurization than other mycobacteria;[27] therefore, it is possible that milk and other foods of animal origin are potential vehicles of transmission of *M. paratuberculosis* to humans. Chiodini et al.[28] reported the isolation of a *Mycobacterium* species from intestinal tissue of three patients with CD, and other reports also have described isolation and identification of *M. paratuberculosis* in individuals with the disease.[37,116,187] Liu et al.,[105] using immunocytochemical techniques, found *Listeria* antigens in intestinal and mesenteric lymph node specimens of 75% of CD patients and *E. coli* and *Streptococcus* species antigens in 57% and 44% of CD patients, respectively. However, the authors suggested that *L. monocytogenes* may have occurred as a secondary infection. Evidence suggesting an etiological role of SLT-producing *E. coli* serotype O2:H5 in UC has been reported,[205] but this finding has not been confirmed. A possible role of bacterial superantigens in the pathogenesis of CD and UC has been described.[14,74,84] Although infectious organisms may be involved in the development of CD and UC, the diseases may have a multifactorial etiology involving dietary, hereditary, microbial, and immunological components.

55.5.5 Hemolytic Anemia

Acute hemolytic anemia, presumably immune-mediated in most cases, has been reported to occur as a complication of bacterial infections due to *Campylobacter* and *Yersinia.*[34,203] Seitz et al.[157] characterized the types of hemolytic anemias in 96 cases in which the hemolytic disease was preceded by a microbial infection. In most cases full recovery occurred within 3 months; however, in 13 patients, the hemolytic disease showed a chronic course. "Warm" or "cold" autoantibodies (antibodies that react best at 37° C or 4° C, respectively) were detected in 17 (17.7%) patients, while in 57.3% of cases the infection-associated hemolytic disease was characterized by alterations of the red blood cell surface due to binding of microbial antigens and in some cases also of complement components.

55.5.6 Miscellaneous

An association between infection with *Yersinia enterocolitica* serotypes and Grave's disease and other thyroid disorders has been suggested.[9,16,210] Patients with various thyroid disorders may possess autoantibodies against thyroid antigens such as thyrotropin (TSH). *Y. enterocolitica* was found to possess a binding site functionally similar to the TSH receptor found in thyroid tissue.[210] Thus, individuals exposed to *Y. enterocolitica* may generate antibodies that cross react with TSH receptors. The antibodies may bind to thyroid tissue, resulting in thyroid hyperactivity. Reports of involvement of specific serotypes of *Y. enterocolitica* are conflicting, and antibodies against *Y. enterocolitica* have been demonstrated in patients with thyroid diseases but also are found in a percentage of healthy individuals.[9] Toivanen and Toivanen[194] concluded that *Y. enterocolitica* does not appear to be a major inducer of thyroid autoimmunity; therefore, further research is needed to determine a role, if any, of *Yersinia* in triggering thyroid disorders.

In adults, the convalescent phase of foodborne illness caused by *Clostridium botulinum* can last for as long as 1 to 2 years. Residual symptoms of botulism include chronic weakness, fatigue, and exertional dyspnea.[177] *Salmonella* infections have caused complications such as orchitis, urinary tract infection, and septicemia.[18,98] Infections caused by antimicrobial-resistant nontyphoidal *Salmonella* strains have increased in recent years, thus rendering difficult the treatment of individuals with complications.[72,98]

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56 The Economic Costs of Foodborne Disease

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56.1 INTRODUCTION

Foodborne disease, defined by the World Health Organization (WHO) as "a disease of an infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water," is recognized as a major international cause of morbidity and economic loss.[105] The WHO estimates that hundreds of millions of individuals worldwide experience diarrheal disease each year, and that many of these cases are foodborne in origin.[106] Most recorded foodborne diseases are microbial in origin and have increased even in industrialized countries. Concern about foodborne diseases is not new, and the fact that they are a worldwide problem of major public health importance and economic loss has been recognized for more than 25 years.[42,82,100,103]

In recent years the economic losses associated with foodborne diseases have attracted increasing attention in North America and Europe; however, it is suspected that the impact of these diseases may be greater in developing countries because of the large numbers of people who are thought to be affected, although few data are currently available to support this. The costs of illness are associated with the investigation, treatment, and prevention of illness, and with loss of productive output, and may affect the whole chain of food production. Thus, the costs of foodborne disease fall both on the public and private sectors and may be extensive both in terms of categories and levels of costs incurred. When outbreaks occur, opportunity costs include public sector resources that are diverted from routine activities often associated with regulation and prevention into the investigation of the cause and the control of disease. In the private sector, financial burdens may be imposed on ill persons and their families as well as on the food industry in general and on the particular company whose product is associated with illness. This chapter examines the rationale behind the economic evaluation of foodborne diseases, discusses the types of cost and their validity, and describes their social and economic implications in terms of estimated national costs of foodborne disease and in the assessment of preventive activities.

56.2 FACTORS AFFECTING INTEREST IN ECONOMIC EVALUATION OF FOODBORNE DISEASE

Economic appraisal of foodborne disease is important in bringing to public attention an essentially preventable illness and in identifying those areas, particularly in the public sector, that are the most costly. However, the most valuable contribution concerns the evaluation of procedures designed to limit or prevent the spread of infection.

Three factors in particular have influenced economists and others to direct their attention to the financial and social impact of foodborne disease. First, it is recognized that foodborne diseases cause significant morbidity and mortality annually, even in developed countries where food safety is well regulated and production and processing standards are protected in law or through codes of practice. [4,22,37,38,41]

Second, there is recognition that the costs to the public and private sectors associated with foodborne illness in general are significant. Although the methods of approach used and types of cost included in published studies vary, the estimated annual costs of salmonellosis and other foodborne disease in the United States (US), Canada, and the United Kingdom (UK) may be in the region of billions of dollars or pounds.[7,39,68,69,79,92,93] The cost estimates given generally have been conservative, but nevertheless indicate that the potential economic impact of these infections at the level of national economy is high. Furthermore, studies that have investigated the impact of human disease-causing organism contamination on the agricultural and food processing industries in the 1970s in Germany and Canada, and more recently in the US, indicate these costs also are high.[23,24,44]

A third, more general factor is growing government concern about the escalating costs of public sector services. The health sector, particularly in industrialized countries, is seen as a major consumer of resources and, therefore, as a legitimate target for both cutbacks and efficiencies.[17,29,31,63,99]

56.2.1 Cost-Benefit Analysis

CBA addresses the question of how to maximize benefits from the resources available and has been used to evaluate the benefits that would derive from the introduction of specific preventive strategies. For example, a CBA of the potential cost reduction associated with human salmonellosis in Scotland that would result from the introduction of universal pasteurization of milk was instrumental in bringing about such legislation.[19] Subsequent studies confirmed the effectiveness of this approach in virtually eliminating milkborne *Salmonella* infection and reducing *Campylobacter* infection.[74] Studies of poultry irradiation in Scotland and England and Wales used a similar methodology to estimate the long-term benefits that would accrue largely from saved health care costs and prevention of lost production due to sickness absence.[79,107] A more recent study of the benefits of HACCP regulations for meat and poultry plants in the US suggested a net benefit if the costs associated with *Campylobacter jejuni/coli*, *Clostridium perfringens*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, nontyphoid *Salmonella* infection, and *Staphylococcus aureus* were reduced by only 20%.[23]

56.2.2 Cost-Effectiveness Analysis

CEA assumes a single outcome, and the evaluation is concerned with meeting this outcome at the least cost. Unlike CBA, CEA can use physical and monetary measures of outcome, but is limited in that difficulties arise when trying to measure more than one outcome. To obviate this, CUA converts outcome measures to a single index such as the quality-adjusted life year (QALY).[99]

56.2.3 Cost-Utility Analysis

The QALY was proposed as a method for estimating the outcomes of health interventions and has been used extensively in CUAs.[108] QALY is a measure of the difference in well-being between a state of perfect health and a state of impaired health. The value of avoiding a debilitating health condition therefore is calculated as the measure of QALYs gained (by prevention or cure) multiplied by the expected remaining years of life. Mauskopf and French[49] have suggested the use of QALYs as a generalized approach for comparing the value of avoiding illness using published data to estimate the value of avoiding morbidity and mortality.

56.2.4 Cost of Illness Approach

The COI approach is more limited in its objectives and usually is concerned with delineating the types and quantifying levels of costs involved, and expresses benefits as costs avoided.[21] The COI approach, frequently adopted in the economic evaluation of foodborne disease, aims to determine the cost of illness to society, usually within a specified time period, although costs avoided may not reflect the real cost of avoiding illness and exclude intangible costs related to pain and suffering. Typically, this approach tends to concentrate on the direct medical and nonmedical costs of illness and indirect costs resulting from lost productivity due to sickness absence, or premature death.

It is difficult to assess the value to society of the activities of the young, the elderly, the unemployed, homemakers, and the disabled. Nonetheless, the value of these activities should be included, and various strategies have been explored to quantify them. These costs can be a significant factor in calculations, although it has been suggested that, overall, the COI approach gives a low estimate of the true costs.[40] Some authors have proposed an approach that combines COI estimates with those for intangible costs estimated using, for example, the willingness-to-pay (WP) approach.[79] While this gives higher overall estimates of the costs of illness, greater care is needed to avoid double counting and inclusion of inappropriate cost categories.

56.2.5 Valuing Health

There is not necessarily a correspondence between the cost of an illness and the value a person would place on not having the illness (ie, avoiding the pain and suffering). A modified WP approach of Landefeld and Seskin[45] provides an alternative method of appraisal of the value an individual puts on avoiding foodborne disease.[70] The WP approach seeks to measure the individual's valuation of health status in terms of what he or she would be willing to pay, or forgo, to avoid risk of death, disability, or illness. This methodology is compatible with economic demand theory in that it reflects the assessment of the individual's health and health needs and will be modified by the ability to pay.[28] Surveys, for example, have attempted to assess consumer willingness to pay extra for safer food; a survey in the UK found that some consumers would pay more for safer food.[3]

Difficulties also arise in relating cost to the severity of illness, which may vary from very mild to death. Some studies have ascribed cases to various categories depending on the level of treatment required; however, even within these categories, there will be a range of severity. The treatment received may be partly a function of actual severity and partly a function of the individual's perception of need; this particularly may be so in individuals who do not require hospitalization. In some studies, the average cost per case may not adequately have reflected the relatively mild illness that probably is experienced by most infected persons. In one publication, however, an allowance was made for mild illness in most people in estimating cost attributed to unreported cases of foodborne illness; in another study, cases were ascribed to one of four severity classes related to death or the level of medical treatment required.[64,65,67]

In the cost benefit study of milk pasteurization in Scotland, estimates were based on UK Ministry of Transport data for assessing injury.[18] The same approach was adopted in the cost analysis of a *Salmonella* outbreak;[64] however, there are difficulties with this approach. How well, for example, do the levels of severity of salmonellosis compare with an accident injury? Little information about the severity of outbreaks of salmonellosis is available. Some information can be derived from the type of medical service sought by individuals, thus "death," "hospitalization," "treatment by a GP," and "no medical attention sought" have been used.[22,64]

In an alternative approach, subjective assessments by patients of illness severity were used to create a severity index that correlated strongly with more objective measures, such as length of illness and use of medical facilities, and with illness-related costs.[79] When applied to national estimates of *Salmonella* costs, this approach yielded, overall, lower average costs than those based on the level of use of medical services and outcome (Table 56–1). This result was attributable mainly to the ability of the severity index to reflect a lower COI of less severe illness, particularly where these related to family costs and loss of production. Public health investigation and treatment costs were the same.

56.3 APPROACHES TO ECONOMIC EVALUATION IN PUBLIC HEALTH

The modern public health function, founded on concern for sanitary hygiene and the control of epidemic disease, and currently defined as "the science and art of preventing disease, prolonging life and promoting health through organized efforts of society," usually has been assumed to be a government responsibility in terms of the enactment of public health laws or regulations, with enforcement at the local level.[2] Expenditure on public health has been regarded as an investment in human capital that benefited the national economy. In an early example, Calkins, commenting on sanitary legislation in England from 1875 to 1890, noted that the death rate in England declined between 1880 and 1889 and that the value of lives saved, based on William Farr's estimated value of life, was $650 million compared with $583.5 million spent on sanitary improvements.[15,30] Calkins concluded: "Thus in ten years the country has more than regained the sum that was spent for sanitary improvements in the fifteen years; and in this calculation nothing figures for maladies avoided . . . spared grief, better health and happier lives." In terms of foodborne disease, economic theory provides a framework for evaluating and comparing the cost implications of, for example, human illness or implementation of a risk-reducing activity, with the benefits of a risk-reduction activity such as milk pasteurization or application of the Hazard Analysis Critical Control Point approach (HACCP) to a production process. The tools of economic evaluation include cost-benefit analysis (CBA), cost-effectiveness analysis (CEA), cost-utility analysis (CUA), and cost-of-illness (COI) studies. These were developed to assist decision making, particularly in the public sector where normal market forces cannot operate.

56.4 CATEGORIES OF COSTS ASSOCIATED WITH FOODBORNE DISEASE

The costs associated with foodborne disease fall under the main headings of public sector costs, costs to ill individuals and their families, costs to the national economy, food industry-related costs, and costs of legal action. Some of the specific costs evaluated in published studies are listed under these categories in Exhibit 56–1.[24,25,68,70,79,95] These may be subdivided into tangible costs, which have a directly measured monetary value, and intangible costs, which do not.

The tangible costs may be presented as direct costs to the affected person or the implicated food producer and include costs associated with providing health care or to an intervention activity. Additionally, tangible indirect costs relate to resource expenditure resulting from participating in an intervention activity or the results of illness, such as income loss because of time off work. Indirect costs to the

Table 56–1 Comparison of Estimated Costs of Human Salmonellosis in England and Wales in 1988 Based on Two Approaches to Identifying Severity of Illness*

	*Public Sector Costs**	*Family Costs**	*Lost Production**	*Total**
Approach I	46.3	64.6	220.2	333.1
Approach II	46.3	22.6	162.2	231.1

* £ million (1988 prices).

Approach I: Severity of illness categorized by type of use of health care service: death, hospitalized, treated by family doctor, medical attention not sought.

Approach II: Severity of illness categorized by patient assessment of severity correlated with the use of medical services.

Source: Copyright © 1993, P.N. Sockett.

Exhibit 56–1 Types of Costs Potentially Associated with Foodborne Illness

Public Sector Costs:
Investigation costs for epidemiology and laboratory analysis
Costs for controlling outbreak including publicity, assisting product recall and premises cleanup, travel
Additional surveillance costs
Treatment

Costs to Ill Individuals or Their Families:
Physician visits
Medication, operations
Hospitalization
Vocational and physical rehabilitation, including home modification
Convalescence
Pain and suffering
Income or productivity loss
Leisure time loss
Home care of ill person
Cancelled arrangements
Purchase of comfort
Education time lost
Risk aversion costs
Averting behavior costs
Travel costs
Value of lives lost (willingness-to-pay portion)

Economy:
Production loss to national economy
Government intervention
Value of lives lost (human capital portion)

Food Industry Costs:
Product recall
Reduced demand for products
Destruction or reprocessing of products
Purchase of new equipment
Design change at plant
Educational programs for staff
Hiring of new/extra staff to deal with crisis
Increased testing of ingredients and final product
Advertising costs to recover market position
Increases of loans
Increased insurance premiums
Possible redundancy with lay-offs or closure
Adverse effect on related business

Legal Action:
Fines, seizures
Jail sentences
Legal suits and out-of-court settlements
Legal costs for lawyers

Source: Data from references 24, 25, 68, 70, 79, 95.

food producer can have a significant effect on business and include loss of public confidence in a particular food that may affect product sales across the food sector, depressing demand and resulting in cutbacks in production. Because some costs could be included in more than one category, particular care is necessary to avoid double counting.

Intangible costs are not directly measurable in financial terms, but they are important to the description of the impact of illness. There is discussion about the methodology of valuing such costs; however, the need to take them into account is agreed on by economists.[54] The intangible costs of illness include pain and suffering and death. Death represents both a loss to the affected person and to the families involved with possible financial implications and a cost to the economy in terms of the loss of the future production potential of the individual. Attempts to place value on pain and suffering have used legal settlements and the WP approach. For example, the UK Department of Transport has used WP studies to create a scale of values for injuries of different severity or disability and death.[27]

56.4.1 Public Sector Costs

Public sector costs include the resources expended on the investigation of cases, the investigation and control of outbreaks, and medical treatment. These activities could impose costs on local public or environmental health authorities; public health physicians; and laboratories and government departments associated with health, agricultural, and environmental issues; provision of medical services, including family practices and hospitals, where these are funded through a government-administered health service. The treatment costs themselves may be defrayed by national insurance schemes across society, as in the UK, or may be borne directly by the individual or the private sector through personal health insurance schemes.

56.4.2 Costs to the Individual and Family

It is important to distinguish public sector treatment costs from those medically associated costs incurred by ill individuals and their families. Direct costs to individuals and their families may include the costs of seeking treatment (eg, costs of travel to doctor, hospital), medicines, costs of care, and the costs of making oneself "comfortable" (eg, purchase of leisure materials and dietary supplements). Indirect costs include disability, loss of income resulting from missed work opportunities or having to change vocation and accept a lower wage, and retraining. Individuals and families may incur considerable loss from cancelled arrangements including holidays and education fees, and may spend large amounts to purchase comfort. This may include replacement of bedding, cleaning and disinfecting materials, dietary supplements, and entertainments. The types of losses and costs incurred by individuals in a study of 1,482 individuals with salmonellosis in the UK in 1988 are shown in Exhibit 56–2.[79]

Changes in lifestyle, particularly eating habits, may result in some foods or restaurants being avoided and more expensive, and perceived "safer" alternatives to the foods implicated in outbreaks may be purchased. Little detailed information on the profile of these costs exists in the context of foodborne illness.

56.4.3 Leisure Time and Activities

Loss of leisure time has been evaluated at the same rate as loss of productive output, allowing different amounts of leisure time for weekdays and weekends, and including a gender and age differential and assumptions about employment status in the calculation of average hourly wage rates.[24,44] This approach assumed that leisure time was valued at least as much as time spent at work.

56.4.4 Value of Life

Estimates of the costs of deaths associated with foodborne disease are difficult to make. Because many of the reported deaths occur in people with underlying medical conditions and the elderly, account needs to be taken of the contribution that the foodborne disease actually made to death. Differentiation should therefore be made between those instances where foodborne disease caused death in a healthy individual, for example, in a community outbreak of botulism or veritoxin-producing *Eschericheria coli* (VTEC) infection, and those situations where infection or intoxication contributed to death, for example, in an outbreak in a home for the aged or on a hospital ward for chronically ill patients. Lastly, foodborne infection or intoxication may be incidental to the final outcome when infection occurs in a terminally ill person and advances the time of death. It therefore becomes necessary to explore these possibilities if a reasonable estimation is to be made of the value of lives lost. Basing the value of lives lost simply on application of a monetary figure to deaths where foodborne infection was recorded would overestimate costs, although this is the approach most commonly adopted simply because of lack of detailed information about outcome and underlying factors. In an analysis of *Salmonella*-related deaths in the UK, there was a significant difference in overall costs of death depending on whether or not cases were differentiated on the contribution of the *Salmonella* infection to outcome.[79]

Two approaches to valuing life have been adopted by most researchers examining the costs of foodborne illness: the human capital (HC) approach, which approximates the effect of permanent or temporary disability or death to the discounted loss of productivity (usually equated to total costs of employment) of the affected individual, and the WP approach (see Section 56.2.5). Both approaches were recommended as appropriate to COI studies.[21] There are, however, drawbacks to these approaches. First, on grounds of social bias, that the HC approach to valuing life on the future production potential of individuals is influenced by age, gender, and race, and therefore values individuals according to these criteria. The WP approach has drawbacks relating to the validity and consistency of the values derived. A combined approach proposed by Landefeld and Seskin gave a midrange estimate of the value of life between that derived by the HC and WP methodologies.[45] Current debate centers around the methodology of valuing life rather than whether or not it is possible.

The value of life, which clearly should be included in cost benefits of preventive measures, will be strongly influenced by the estimated number of lives lost. For example, in cost-benefit studies of milk pasteurization and poultry irradiation in Scotland and of preventing *Salmonella napoli* infection following an outbreak in England in 1982, the results were significantly influenced by the assumptions made about the numbers of deaths and the adopted

Exhibit 56–2 Types of Costs Incurred by Families in a Study of 1,482 Cases of Salmonellosis in the UK in the Period 1988–1989[79]

ADDITIONAL EXPENDITURE	
Seeking treatment:	Travel to and from family doctor and hospital Refreshments Prescribed medicines Nonprescribed remedies
Comfort:	Clothes and toiletries for use in hospital Leisure items (books, games, videos) Dietary supplements (food and drink) Communications (telephone calls) Replacement clothing and bedding Travel by visitors and caregivers Additional home heating
Hygiene:	Cleaning materials Disinfectants and bleach
UNEXPECTED LOSSES	
Holidays:	Time lost by ill person and accompanying friends and family Curtailed or extended stay Insurance excess Excursions not taken
Other:	Cancelled or postponed arrangements such as theater trips, weddings, family parties Prepaid child-care and education fees not used Unused travel season tickets or vouchers

Source: Copyright © 1993, P.N. Sockett.

method of valuing life.[19,65,107] This is also clearly seen in the significant contribution that high estimates of deaths made to the overall costs of foodborne disease in the US.[67,68]

56.4.5 National Economy

Lost production has proven to be a significant cost to the economy. These costs may be generated by absence of the affected person from work due to illness or premature death and by family or friends taking time off to take care of those who are ill.[65] The lost productive output was estimated to be up to half of all the tangible costs of two of three outbreaks and a study of national *Salmonella* costs in the UK between 1981 and 1988, although the proportion varies by outbreak (Table 56–2).[18,56,80,107] In these studies, production loss was calculated on production potential of the individual being equivalent to at least the gross daily employment costs of that individual.[55] This is likely to be an underestimate because it does not account for the profit of a firm that results from the employees' activities. Aggregated employment costs were subject to the proportion of working adults involved in an outbreak, and were correspondingly lower in a *Salmonella* outbreak in 1982 when more than 60% of cases were in children. Further debate may be necessary to allow for differences in wage rates that relate to age, gender, and status within a company. For example, labor market data may undervalue a woman's contribution to the labor force. The removal of this gender bias significantly increased productivity losses.[64]

There is debate about the extent to which absence from work affects productivity, and it could be argued that the work will be made up either by slack in the system or by imposing extra work on other members of staff. It also could be argued that there is

Table 56–2 Lost Production as a Proportion of Total Costs in Three Salmonella Outbreaks and a National Study of Salmonellosis Costs in the UK

Type of Study	*Year*	*Total Cost (£)*	*Production Lost (£)*
Salmonella outbreak	1981	84,089	36,864 (44%)*
Salmonella outbreak	1982	504,808	15,356 (3%)*†
Salmonella outbreak	1984	8,364	3,270 (39%)*
Study of sporadic *Salmonella* cases	1988	996,350	507,555 (51%)*

* percentage of total costs expressed as 1988 prices.
† 60% of cases were in children aged under 15 years.

Source: Data from references 18, 56, 64, 80.

no productivity loss if there is unemployment in the system on the assumption that replacement staff can be taken from the pool of unemployed. For foodborne disease, the presence or absence of unemployment is largely irrelevant as absence, in most cases, will be short and the costs of a company seeking alternative employees prohibitive.

56.4.6 Losses within the Food Industry

Losses may be incurred by the food industry when a product is implicated as a source of illness or when contaminated products are withdrawn from sale. Costs borne by the food industry at the primary producer (farm), manufacture, import/export, processing, or service and retail stages include direct and indirect costs. Immediate loss of business may occur when a food service outlet or manufacturer is identified as a source of infection. The cost to the company may be substantial; consumers may switch to an alternative brand or product, resulting in severe financial effects on the firm. For example, after a manufacturer's withdrawal of infant feed products in the UK in December 1985 following an outbreak of *S. ealing* infection, parents switched to other brands and there was a significant loss in the sale value of the company.[73] The effect on the wider economy may be minimal, however, unless long-term unemployment results from, for example, loss of public confidence in a product or plant closure.

Where a specific product is implicated as a cause of illness, it is liable to be recalled, held for the period of investigation, and eventually destroyed. Recall may be expensive and involve national and local government agencies as well as the food industry, and product destruction must be counted as an outright loss.[77] Recall costs in seven outbreaks were US $83,000 to $9.8 million and were 24% to 99% of the total outbreaks costs (Table 56–3).[89] Withdrawal of a product or closure of a restaurant may result in a decline in public confidence, which may then take years to recover and may affect sales of similar products of different brands. For example, decline in public confidence had a major impact on the manufacturer in the *S. ealing* outbreak mentioned above. By 1989 (4 years after the outbreak), the company had recovered only half of its former market share; one production unit was closed and about 200 jobs had been lost.[85] Similarly, sales of corned beef in the UK were depressed for many years following a typhoid outbreak associated with canned corned beef in 1964, and sales of canned salmon in the UK were affected for several years after cases of botulism were associated with canned products from North America in 1974.[89] Even for food service establishments that have been implicated in outbreaks, the costs may be high. In a study of 17 outbreaks, mainly in North America, the median cost per case was $788 in 1983 dollars.[88]

Other direct costs may result from fines and the need for renovation and cleaning of premises, replacement of defective equipment and training of present or new staff, and promotional costs. It could be argued that at least some of these costs, including items of capital expenditure on new equipment, may have occurred anyway and that the outbreak only hastened the expenditure. However, because it is unlikely to be known when this expenditure would take place and the producer would want to restart production as quickly as possible, they arguably could be included in the direct cost of the outbreak.

Staffing costs may include salaries of staff suspended because they are excreting a pathogenic organism, or salaries of staff laid off while the premises

Table 56–3 Food Industry Losses in 9 Selected Outbreaks of Foodborne Illness or Recalls in Various Countries

Agent	*Product*	*Number of Cases*	*Loss of Product ($)**	*Recall Costs ($)**	*Production Stoppage ($)**	*Total ($)**
S. aureus	cheese	15	48,700	468,700	84,000	601,400
Salmonella	cocoa	110	NA	83,000	NA	>83,000
Salmonella	chocolate	200	48,465,000	9,872,300	NA	>58,337,300
Salmonella	chicken	450	1,372,000	included in loss	included in loss	1,372,000
Salmonella	diet drink	0	1,548,000	484,000	NA	>2,032,000
S. typhi	corned beef	503	160,000,000	included in loss	NA	>160,000,000
S. aureus	corned beef	80	NA	2,744,000	NA	>2,744,000
C. botulinum	canned salmon	4	NA	5,600,000	included in recall	>5,600,000
C. botulinum	canned salmon	2	4,285,000	825,000	1,540,000	>6,650,000

* All amounts shown in US $ (1983 prices).
NA: not available.

Source: Adapted with permission from E.C.D. Todd, Economic Loss from Foodborne Disease and Non-Illness Related Recalls Because of Mishandling by Food Processors, *Journal of Food Protection*, Vol. 48, pp. 621–633, © 1985, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

are closed or being renovated and cleaned. Alternatively, costs could arise from overtime or temporary employment of additional staff to cover for those suspended or to help in cleaning.

56.4.7 Government Financial Commitment to Public Food Safety, and the Industry Sectors

Further costs to the economy can occur when governments act to intervene by increasing surveillance or research in the area of foodborne disease or to support a sector of the food industry affected by a lack of product confidence. For example, the UK government, in 1989, instituted a series of inquiries in response to public concern over *Salmonella*-contaminated eggs. These, and concerns over listeriosis, led to formation of an Advisory Committee on the Microbiological Safety of Food (ACMSF), and a number of working groups were convened to examine all aspects of food production and to commission research into product safety and disease surveillance.[1,2] In addition, the UK government pledged £7 million to support, through compensation, sectors of the egg industry affected by the government policy of slaughtering infected layer flocks.[51] More recently, the UK government has acted to support the UK beef industry with compensation following policies to "protect" the public from the consequences of bovine spongiform encephalopathy (BSE).[53] Other examples included payment of US $5 million (1983 prices) by the Canadian and Alaskan state governments in promotional costs to support the salmon industry following public loss of product confidence after a botulism incident in Belgium in 1982, and payment of about US $3.6 million by the Canadian government to support a chocolate producer following association between their products and *Salmonella* infection in 1974, thereby offsetting costs of unemployment.[89]

Indirect industry-related costs may include a loss in the actual value of the company; for example, through a decline in share values. The company in the *S. ealing* outbreak had been on offer for sale at £40 million but following the outbreak eventually was sold for £18 million, a £22 million loss in its value.[73,78] Industry losses in the US in the 1980s resulting from *Listeria* contamination in commercial foods were estimated to be more than US $9.3 million or about 0.1% to 0.2% of the value of sales.[72] Between 1986 and 1988, to reduce contamination with *Listeria* spp., food processing plants changed production lines, increased laboratory testing, performed major cleanups and increased sanitation, replaced equipment, and educated workers and management.

56.4.8 Legal Costs

Legal damages may result from government regulatory actions and legal suits initiated by individuals or by companies who feel that a warranty or contract has been broken. The costs involved are difficult to obtain, but sometimes are mentioned in newspaper articles. Some of these legal costs can be huge and may account for a major proportion of total costs to a company of an outbreak where they have been included.

Products that appear to be in violation of an act or regulation may be seized and the companies producing or selling them may be fined; licenses may be withdrawn and officials jailed. For instance, in Wisconsin in 1965 and Quebec in 1977, cheese worth US $124,400 and US $320,000, respectively, was seized and destroyed because of the presence of *S. aureus* enterotoxin.[89] Company fines have ranged from $500 to $12,000 in the US, and owners of violative companies may be jailed or put on probation as well as fined as occurred with the 1985 *Listeria monocytogenes* outbreak due to a Mexican-style cheese in California and an *E. coli* O111 outbreak associated with mettwurst in New South Wales, Australia. In the UK, meat producers failing to comply with meat hygiene or meat processing regulations designed to limit the threat of BSE transmission to humans face severe financial penalties.

Litigation is sought when an individual claims to have a just cause, irrespective of violation of regulations or codes of practice. Those claims most frequently documented are associated with large outbreaks that receive wide publicity. Settlements can be awarded in court decisions, out-of-court settlements, or through worker compensation boards. Reasons given for taking legal action include illness and loss of job when individuals are identified as carriers in hospital or food retail situations. Ten settlements in the US and Canada following outbreaks ranged from $12,306 to $1.9 million (1985 US and Canadian dollar values) and from 1.2% to 95.3% of the total incident costs (Table 56–4).[90] Settlements were much higher for two *S. aureus* outbreaks (Can $1.5 million to $1.7 million) and two botulism outbreaks (US $0.6 million to $2.0 million); these ranged from 22% to 84% of total incident costs.

Following a multistate outbreak of *E. coli* O157:H7 infection in the US in 1993 (not included in the table), millions of dollars have been paid for medical and hospitalization costs and to franchisees who lost money due to bad publicity. A few individual settlements were publicized, such as a high figure of US $15.6 million being given to one chronically ill child.[9] The final total will be in excess of US $100 million (C. Bartleson, personal communication). The payment made by a major UK airline following a *Salmonella* outbreak in 1984, which resulted in more than 600 illnesses in passengers and two associated deaths, was in the order of several million pounds sterling (British Airways press office, personal communication).[12] In 1991 a farmer in England was ordered to pay £33,000 damages to the families of two customers who died from *Streptococcus zooepidemicus* infections after drinking contaminated raw milk.[86] Settlements thus vary considerably in amount, but generally, the more severe the illness, the larger the settlement. Furthermore, it is probable that in most instances only those willing to take legal action will normally get any kind of settlement, although the use of class action suits may increase the number of persons receiving compensation.

Companies also will sue other companies where contaminated ingredients have resulted in illness or product loss. For instance, in 1978 a club sued a bean canner for allegedly supplying beans that contained *Clostridium botulinum* toxin that caused 34 cases of botulism. The club, which had been sued by the victims, won US $1.5 million from the canner in 1981.[48,89] Typically, insurance companies pay most of court and out-of-court settlements; however, this potentially results in increased insurance premiums not only for the producer or retailer involved, but also for the industry if sufficient claims are made. The effect of legal action may not be represented adequately by the eventual awards; the process may take several years to complete and cause considerable stress to all concerned.

56.5 ESTIMATED CASES AND DEATHS DUE TO FOODBORNE DISEASE

Case estimates are important for economic assessment because numbers of cases and deaths affect the total cost of foodborne disease. These estimates often are calculated as a multiple of an average cost per case. Many industrialized countries report cases and outbreaks of foodborne disease, but the type and extent of reporting varies considerably. On a population basis, cases may range from 7 to 123 per 100,000 population, and the ratio of cases to outbreaks shows a similar amplitude indicating that, in some countries, only moderately large or large outbreaks are included in the final figures, although smaller outbreaks may have been investigated. In contrast, relatively few countries have attempted to estimate the number of cases and so determine the associated annual economic burden. US figures, based on extrapolation of sentinel county studies, health surveys, and

Table 56–4 Liability Costs in 10 Court and Out-of-Court Actions Following Foodborne Disease Outbreaks in North America

Agent	*Location*	*Number of Cases with Settlement per Ill People*	*Settlement Costs ($)**	*Legal Costs ($)**	*Total Liability Costs ($)**	*Liability Costs as a Percentage of Total Costs*	*Average Settlement per Person ($)**
Salmonella	Canada	23 of 23	185,293	7,878	193,801	95.9	8,056
S. aureus	Canada	1 of 26	1,522,048	NA	>1,522,048	84.0	>1,522,048
Salmonella	Canada	14 of 119	48,837	35,101	83,938	67.6	3,488
Salmonella	US	115 of 234	380,058	52,458	432,516	87.9	3,305
S. aureus	Canada	5 of 19	1,719,829	NA	>1,719,829	56.2	>343,966
Salmonella	Canada	76 of 196	261,219	NA	261,219	47.4	3,437
C. botulinum	US	59 of 59	332,760	270,650	603,410	41.4	5,640
C. botulinum	US	8 of 34	998,079	759,945	1,957,869	21.7	124,760
Salmonella	Canada	1 of 7	7,648	4,658	12,306	11.5	7,648
Salmonella	Canada	11 of 264	33,329	20,622	53,951	1.2	3,030

* Costs shown in US $ and Can $ as appropriate (1985 prices).
NA: not available.

Source: Adapted with permission from E.C.D. Todd, Legal Liability and Its Economic Impact on the Food Industry, *Journal of Food Protection*, Vol. 50, pp. 1048–1057, © 1987, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

expert opinion put estimated case numbers (1.4 to 33 million annually) much higher than the recorded numbers from the passive reporting system (about 18,000 annually); and the number of people estimated to have died from foodborne disease to be between 500 to 9,000 annually.[22] Estimates of foodborne disease in Canada, including diseases associated with seafood toxins, suggested about 2.2 million cases each year.[92,93] Both sets of data have been determined by accumulating estimates for specific foodborne diseases. For a more detailed discussion of case estimates, see Chapter 37.

Bennett et al., in estimating the number of people who died in an outbreak of foodborne illness in the US, assumed that the proportion of cases to deaths was the same for both known case numbers and extrapolated numbers with a total number of deaths close to 9,000.[11] Other researchers consider that there are no deaths in the extrapolated case numbers or that the proportion of deaths is much reduced; this would therefore result in lower estimates of the cost of illness.[22,79,96,101] Recently, the widely quoted 9,000 death figure for the US has been criticized as being too high.[102]

56.6 NATIONAL ECONOMIC STUDIES OF FOODBORNE DISEASE

A relatively small number of studies of the national costs of foodborne illness in general or specific foodborne illnesses are reported in the literature, largely from North America and Europe; however, five estimates of national costs related to disease problems in Asia and Latin America also are noted in this section. The costs derived in these studies were dependent on estimates of the annual numbers of cases and on cost-per-case data derived from studies of outbreaks. Most have concentrated on estimating costs associated with medical treatment and productivity losses; many categories of costs were excluded, and the amounts derived, therefore, represented the minimum costs of disease. Exceptions to this were a national study of *Salmonella* infections in England and Wales in 1988–1989 that was based on a detailed survey of individual cases, and national studies of *Salmonella* costs in Germany and Canada that sought to quantify a wide range of cost categories.[24,44,80] These are reviewed in detail in the next section.

The range in categories of costs included in various studies, differences in the range and types of agents included, methodologies used in extrapolating to national totals, and differences in population sizes and economic activity make it difficult to compare costs from one country with another. Limitations on the cost categories included and a conservative approach to estimated numbers of cases indicate that the apparently large costs reported may underestimate the true impact of these diseases. Some of the more widely reported national studies of foodborne disease in general or of specific agents are reviewed below.

56.6.1 Canada

The cost of an estimated 2.2 million cases of bacterial foodborne disease per year from 1978 to 1982 in Canada was Can $1.175 billion, of which salmonellosis, *S. aureus* intoxication, and *E. coli* O157:H7 infections were the most expensive (Table 56–5).[93] The estimated 627,200 *Salmonella* cases cost Can $846 million at a cost per case of Can $1,350. This compared with a subsequent estimate of 847,400 cases costing Can $203.2 million at a lower cost per case of $240.[25]

Botulism in native peoples from improperly fermented fish or marine mammal products was estimated at Can $2 million annually ($7,200 per case and $100,000 per death). These relatively high costs reflected the costs of evacuation of patients from remote communities, hospitalization, and illness investigations in six incidents between 1984 and 1986.[91] Outbreaks arising from commercially produced food contaminated with *Clostridium botulinum* were expensive, costing from Can $1.5 to $160 million;[89] therefore, it is not surprising that the cost of botulism in Canada was estimated at Can $17 million annually with the highest cost-per-case figure, Can $340,000, of any agent. Other agents included an estimated 8,400 foodborne cases of giardiasis, trichinosis, and taeniasis that were estimated to cost Can $34.8 million or $4,140 per case; most illness was associated with the first two parasitic diseases.[93]

In addition to microbial food poisoning, assessments of the number of cases and costs related to an estimated 3,150 plant poisonings (Can $1.0 million), 29,400 chemical poisonings (Can $8.4 million), and 4,370 illnesses associated with consuming animal products—mainly seafood toxins—costing Can $10.3 million have been made.[93]

The estimates of illnesses associated with seafood toxins recently have been revised to indicate fewer cases and a lower overall cost of Can $820,000.[98] A further Can $4.7 million were related to fish and shellfish monitoring and analysis to prevent illness

Table 56–5 Estimated Annual Numbers of Cases and Deaths and Costs of Foodborne Disease in Canada

Etiological Agent	*Reported Foodborne Cases**	*Estimated Cases†*	*Estimated Deaths*	*Estimated Costs ($ million)***	*Average Cost per Case ($)***
Salmonella	1,792	627,200	8.1	846	1,350
S. aureus	291	101,850	1.2	134	1,320
E. coli 0157:H7	37	12,950	11.6	47	3,600
C. perfringens	633	221,550	0.9	41	190
L. monocytogenes	8	2,800	4.5	34	12,110
C. botulinum	5	50	1.7	17	330,000
Campylobacter	47	16,450	0.3	15	925
B. cereus	66	23,100	0	10	430
Shigella spp.	54	18,900	0.5	8	400
Other microbial	136	47,700	0.4	25	524
Subtotal All microbial	3,069	1,072,550	29.2	1,176	1,100
Seafood toxins	25	2,970	0.6	10	3,000
Other agents‡	121	42,350	0.5	44	1,039
Unknown agents	2,975	1,041,250	1.3	105	101
Total	**6,190**	**2,159,120**	**31.6**	**1,335**	**620**

* Mean of years 1978–1982.

** Costs based on Can $ (1985 prices).

† Investigated foodborne disease incident cases x 350, except for *C. botulinum* and paralytic shellfish poisoning toxins, which were considered to be x 10 because they produce distinctive symptoms.

‡ Parasitic, plant, chemical, and animal other than seafood toxins.

Source: Adapted with permission from E.C.D. Todd, Preliminary Estimates of Costs of Foodborne Disease in Canada and Costs to Reduce Salmonellosis, *Journal of Food Protection*, Vol. 52, pp. 586–594, © 1989, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

from commercial products. One amnesic shellfish poisoning episode with at least 107 cases and 3 deaths caused by consumption of domoic acid in mussels in 1987 was estimated to have cost Can $8.4 million for illnesses, deaths, and lost business through adverse publicity. Such was the devastating impact of this intoxication on the lives of those affected and the local economy that monitoring of shellfish was enhanced to prevent further illness.[16,97]

56.6.2 United States

A comparison of two sources of estimated annual cases and costs of foodborne disease in the US has been made (Table 56–6).[95] The 6.3 million cases and 7,041 deaths at an estimated cost of US $5.8 billion estimated by Roberts[68] were significantly lower than the 12.6 million cases and 523 deaths costing US $10.7 billion proposed by Todd.[94] The difference reflected the inclusion of 7 million cases from seafood toxins, bacterial, viral, parasitic, chemical, plant, and unknown agents not considered by Roberts and marked variations in estimates for number of cases and deaths from specific pathogens; Roberts used COI estimates including medical and productivity costs, whereas Todd added some industry and legal costs.

Revised estimates published in 1996 for a limited group of bacterial pathogens proposed totals of 3.6 to 7.1 million cases and 1,600 to 6,500 deaths, with associated costs of US $2.9 to $6.7 billion for six diseases (Table 56–7).[13] The estimates were based on medical costs and costs of lost productivity; however, there were differences in how they were evaluated. Medical and loss of productivity costs for *Staphylococcus aureus* and *Clostridium perfringens* were calculated on the basis of annual cases multiplied by average annual costs for a previous study

Table 56–6 A Comparison of Preliminary Annual Case and Cost Estimates for Specific Foodborne Diseases in the US

Etiology	*Estimated Costs*		*Estimated Deaths*		*Estimated Costs ($ million)**	
	Roberts	*Todd*	*Roberts*	*Todd*	*Roberts*	*Todd*
Salmonella (nontyphi)	1,920,000	2,960,000	1,920	32	1,613	5,053
S. aureus	1,513,000	1,155,000	1,210	6	1,090	1,919
L. monocytogenes	1,581	25,000	433	68	256	396
C. jejuni/coli	2,100,000	170,000	2,100	1	1,764	198
E. coli (non 0157:H7)	50,000	44,000	100	<1	72	176
C. perfringens	10,000	652,000	10	8	61	156
Y. enterocolitica	3,250	20,000	2	<1	1	138
C. botulinum	180	270	7	4	5	110
E. coli 0157:H7	not calculated	25,000	not calculated	6	not calculated	106
Shigella spp	90,000	163,000	180	2	130	80
Vibrio spp	10,025	42,000	400	32	239	64
B. cereus	5,000	84,000	0	0	1	46
Streptococcus	500,000	52,000	150	<1	210	35
Brucella spp	20	1,000	0	<1	1	9
S. typhi	480	240	29	<1	16	5
Other enteric bacteria	50,000	107,000	500	11	306	90
Subtotal of All Bacteria	6,253,536	5,500,510	7,041	170	5,765	8,581
Seafood toxins	not calculated	58,260	not calculated	3	not calculated	158
Viral, parasitic, chemical, plant agents	not calculated	1,805,500	not calculated	327	not calculated	1,260
Unknown agents	not calculated	5,217,000	not calculated	23	not calculated	670
Total	**6,253,536**	**12,581,270**	**7,041**	**523**	**5,765**	**10,669**

* All costs expressed in US $ (1985 prices).

Source: Data from T. Roberts, Human Illness Costs of Foodborne Bacteria, *American Journal of Agricultural Economics*, Vol. 71, pp. 468–474, © 1989, American Agricultural Economics Association, and E.C.D. Todd, Preliminary Estimates of Costs of Foodborne Disease in Canada and Costs to Reduce Salmonellosis, *Journal of Food Protection*, Vol. 52, pp. 586–594, © 1989, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

updated to 1993 prices.[68] For *Salmonella, Campylobacter, E. coli* O157:H7, and *Listeria*, cases were divided into one of four severity groups to which costs were then applied before aggregation. For the latter two diseases, some chronic implications also were included in the estimates. In addition, recent estimates of *Campylobacter*-associated Guillain-Barré syndrome in the US were between $0.26 and $1.86 billion annually. Combining this total with estimated costs of foodborne campylobacteriosis indicated an annual cost of $0.8 to $10.1 billion.[13,14] These results suggest the sequelae of foodborne infections may have a significant, and as yet undetermined, impact on the overall cost of foodborne disease.

The number of annual foodborne cases and deaths presented in Table 56–7 was based on current best estimates, and the authors point out that the estimates will be subject to revision as improved data become available. For each pathogen, only the proportion of all cases that were foodborne are shown in Table 56–7; this proportion varies from 17% for *Staphylococcus aureus* infections to 100% for *Clostridium perfringens*. The authors acknowledge that the estimates given undervalue the real economic cost of foodborne illness to society. First, a number of significant human pathogens are excluded from the COI analysis, and second, intangible costs such as pain and suffering and lost leisure time of the affected people and their family, food industry related costs and government resources used to investigate, and control incidents were not included. However, the template presented provides a consistent approach for estimating national costs.

Table 56–7 Annual Estimated Cases and Costs for Selected Foodborne Pathogens in the US

Pathogen	*Foodborne Cases*	*Foodborne Deaths*	*Human Capital Approach ($billions)**	*Labor Market Approach ($billions)**
C. jejuni or *coli*	1,100,000 to 7,000,000	116 to 564	0.8 to 5.7	1.6 to 10.1
C. perfringens	10,000	100	0.1	0.5
E. coli 0157:H7	16,000 to 32,000	63 to 156	0.16 to 0.3	0.3 to 0.7
L. monocytogenes	928 to 1,767	230 to 485	0.1 to 0.3	1.3 to 2.4
Salmonella	696,000 to 3,840,000	870 to 1920	0.9 to 3.6	4.8 to 12.3
S. aureus	1,513,000	454	1.2	3.3
Toxoplasma gondii	*260*	*40*	*3.3*	*7.8*
Total	**3,309,000 to 12,400,000**	**1,900 to 3,700**	**6.6 to 14.5**	**19.6 to 37.1**

* US $ (1996 prices).

Source: Adapted from J.C. Buzby and T. Roberts, Guillain-Barré Syndrome Increases Foodborne Disease Costs, *Food Review*, Vol. 20, pp. 36–42, © 1997, Economic Research Service, U.S. Department of Agriculture.

A way of combining costs of sporadic and outbreak cases and the effect of severity, sequelae, and deaths has been suggested.[95] If it is assumed that there are an estimated 2,000,000 cases of salmonellosis per annum in the US, the cost could be US $1,026 per case for a total of US $2.1 billion (Table 56–8). This approach could be expanded to other diseases.

56.6.3 Croatia

An alternative method was proposed for estimating the national costs associated with about 8,500 cases of foodborne illness, most due to *Salmonella* infection, that occur annually in Croatia.[61] No extrapolation was made to account for nonreported cases. Published foodborne illness costs in Canada, Sweden, the UK, and the US were multiplied by the gross national product (GNP) per capita in Croatia and divided by the GNP per capita in these other countries. Thus, a salmonellosis case costing US $1,350 in Canada or the US would cost only US $280 in Croatia. If this assumption is correct, the estimated cost of the 8,500 cases in Croatia would be about US $2 million. This approach may be applicable for other countries that do not have the resources to determine actual costs, although the GNP may not be the only factor to consider.

56.6.4 Cholera in Peru

The cholera outbreak, caused by *Vibrio cholerae* O1, that has been spreading through South and Central America since 1990, is reported to have had a devastating impact on the economy of Peru, the first country to be affected. Cholera now is endemic in most Central and South American countries with more than 600,000 cases and 5,000 deaths reported from 20 countries. In Peru, there were nearly 500,000 cases in 1991, with foods and beverages from street vendors being major factors in infecting the population.[62]

The economy of Peru was affected by loss of revenue from tourism and food, lost productivity from absence from work, and increased medical costs; in addition, local food industries have suffered depressed demand for their products.[59] Total costs for 1991 were determined to be $495 million, of which the greatest losses were for reduced tourism (US $147 million), local fishery sales (US $32 million), exports (US $28 million) and street food vending (US $16 million), health care for cholera patients (US $33 million), and lost productivity due to illness and deaths (US $26 million). No estimate was made for lost leisure time, cost of self-medication, and drugs purchased. However, there was also a prediction that future deaths would cost the economy US $234 million in lost production. Other Latin American countries probably are experiencing similar but lesser economic effects; the Peruvian situation received high and damaging publicity throughout the world. Cholera is expected to remain endemic in most of Latin America for many years to come.

56.6.5 *Staphylococcus aureus* in India

An outbreak of *S. aureus* intoxication in Hyderabad City, India, affected 64 people for an estimated cost of US $673.[83] This is a low amount compared with industrialized countries, but not for residents of a developing nation. About 1,000 *S. aureus*

Table 56–8 Combined Costs per Annum of Sporadic and Outbreak Cases of Salmonellosis in the US[+]

Type of Case	*Establishment Responsible*	*Severity of Illness*	*Number of Cases*	*Costs/ Case ($)*	*Total Costs ($ million)*	*Cumulative Costs/Case ($)*
Sporadic cases and small family outbreaks		Mostly unknown	Hospitalized			
		(1.76%)[b]/severe[c]	29,920	5,330[a]/7,068[b]	160[a]/212[b]	
		Physician visits				
		(5.04%)[b]/moderate[c]	85,680	830[a]/939[b]	71[a]/80[b]	
		No medical care				
		(93.2%)[b]/mild[c]	1,584,400	270[a]/234[b]	428[a]/371[b]	330
			1,700,000		659[a]/663[b]	
Outbreak (15%)	Food service (14.25%)	Not determined	285,000	1,467[c]	418	
	Food processing (0.75%)	Not determined	15,000	16,600[d]	249	
			300,000		667	664
Sequelae (2%)		Chronic disease	40,000	6,700[e]	268	798
Value of lives lost (0.05%)			1,000	456,000[f]	456	1,026
Total			2,000,000		2,052	

[+] US $ (1991 prices).

[a] values of Roberts.[68]

[b] values of Mauskopf and French.[49]

[c] median cost per case for outbreaks mishandled by food service establishments.

[d] median cost per case for outbreaks mishandled by food processors.

[e] Mauskopf and French.[49]

[f] Landefeld and Seskin.[45]

Source: Data from T. Roberts, Human Illness Costs of Foodborne Bacteria, *American Journal of Agricultural Economics*, Vol. 71, pp. 468–474, © 1989, American Agricultural Economics Association.

cases are recorded each year in the same city.[83] If it is assumed that only 1 in 100 cases are reported (possibly a low estimate for a mild disease), and the cost per case is the same as in the outbreak (US $11.60), the estimated 100,000 *S. aureus* cases in Hyderabad City annually cost about US $1,160,000. Extrapolation to the whole country suggests annual costs for an estimated 34,000,000 cases would be US $340 million.

56.6.6 Opisthorchiasis in Thailand

The liver fluke *Opisthorchis viverrini* is the main cause of foodborne parasitic infections in Thailand that arises from the consumption of raw or undercooked fish. As many as 60% of the work force is estimated to be infected in the northern part of the country. On the assumption that workers are off work for a total of 9 days each year from three separate infections, and that they require hospitalization and drugs, the overall cost of illness is thought to be about US $99.9 million annually.[71]

56.6.7 Neurocysticercosis in Latin America

The estimated annual costs of this disease were reportedly US $85 million in Brazil and $195 million in Mexico, indicating the significant impact of foodborne disease in developing countries as well as in industrialized nations.[71]

56.6.8 Fungi and Aflatoxins in Southeast Asian Countries

Chronic effects of microbial agents are not usually estimated; however, an attempt was made to estimate the effects of aflatoxins in three Southeast Asian countries.[47] The cost of premature death from primary liver cancer and disability and morbidity from liver cancer attributable to aflatoxins in corn and peanuts was estimated to be Australian $228.5 million in Indonesia, A $39.0 million in the Philippines, and A $23.7 million in Thailand. The difference in costs is proportional to the amount of aflatoxin ingested.

56.7 NATIONAL ESTIMATES OF SALMONELLOSIS COSTS

National estimates of the economic impact of salmonellosis have been reported from countries in Europe and North America since the late 1970s. This has been largely in response to the recognition of this agent as a major cause of foodborne illness in these countries, together with recognition of the association between the human illness and widespread contamination of poultry and poultry products and other meats.

Two studies of the national costs of *Salmonella* infection carried out in the late 1970s were reported from Germany and Canada; estimated annual costs of human illness were DM108 million and Can $84 million respectively.[24,44] These studies presented a detailed analysis of medical and productivity losses but differed in other categories of costs including estimations for some intangible costs such as loss of leisure time and pain and suffering. Both studies estimated further costs of DM131 million and Can $3 million that fell, respectively, on the poultry, cattle, and pork industries in Germany and the poultry industry in Canada. These losses were attributed to the results of *Salmonella* infection in animals or poultry, leading to slaughter, quarantine, or treatment. The German study also presented an analysis of the potential benefits of preventive activities designed to reduce *Salmonella* infection in animals and humans.[44] More recent estimates of human salmonellosis costs in Canada of Can $203 million to Can $846 million and the US of US $0.6 billion to US $3.5 billion indicated the earlier estimates were low.[25,67,93,94]

56.7.1 England and Wales

A detailed study of the costs of salmonellosis was conducted in England and Wales from 1988 to 1989.[80] The aims of the study included the construct of a cost profile of salmonellosis and to provide a basis for estimating national costs that could be used in economic appraisal of preventive strategies. The costs of illness, including direct costs of investigation and treatment of cases, as well as costs to the affected people and their family and lost production from sickness-related absence from work, were obtained via questionnaires completed by individuals with laboratory-confirmed infection and the investigating environmental health officer.

The study identified tangible costs of £1 million (1988 prices) associated with the 1,482 cases in the sample. Public sector costs accounted for 39% of the total, just under half of which was for the investigation of cases. Costs to families and the economy accounted for the remaining 61%. These included production losses associated with sickness-related absence from work that dominated society costs and were more than half of the total costs identified.

To estimate the national burden of disease, the identified costs were extrapolated using the results of a survey that included information about the incidence of gastroenteritis in a randomly selected sample of adults.[52] The results suggested that only one in six people affected consulted a doctor and that only 1 in 38 of all cases was reported centrally. Applying these multiplication factors to the approximately 23,000 reported *Salmonella* cases in 1988 suggested that about 874,000 people were affected, including 149,000 who sought medical treatment and 4,255 cases admitted to a hospital.[79] Costs were related to the severity of illness by using two approaches (see Section 56.4): approach I was based on demand for type of health care;[68] approach II used a severity index that related use of services and length of illness to the patient's subjective appraisal of the number, type, and severity of symptoms.[78,79] Both approaches showed a relationship between the increase in severity of illness and increased use of services and cost of illness.

Total tangible costs estimated for England and Wales in 1988 were £331 million (approach I) and £231 million (approach II) (1988 prices) (Table 56–1). The lower total for approach II reflected the wider range of severity identified and the lower costs per case for the least severely ill. Inclusion of intangible costs would have added a further £32 million to £119 million to these totals. The costs of human *Salmo-*

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nella infection for England and Wales in 1988 were thus estimated to be between £263 million and £450 million (1988 prices).[79]

56.7.2 Scotland

A large outbreak of salmonellosis in 1981, including 654 cases and 2 deaths arising from consumption of raw milk, cost £1.3 million in direct and indirect costs.[19] This led to a cost-benefit study that recommended mandatory pasteurization of milk that was initiated in Scotland in 1983.[18]

56.7.3 Sweden

In Sweden, 6,043 cases of salmonellosis were reported in 1987 at an estimated cost of US $362,000; 212 of these cases were attributed to eating domestically produced poultry.[58] In a second study, the direct and indirect costs of an estimated 348 domestically acquired *Salmonella* cases arising from poultry, cattle, or swine in 1992 were estimated as SEK 4.9 million (Swedish crowns) (A. Engvall, 1993, personal communication).

56.8 COSTS AND BENEFITS OF PREVENTING FOODBORNE INFECTION

The value of economic appraisal of foodborne disease is three-fold. First, in bringing to public attention an essentially preventable illness; and second, in identifying those areas, particularly in the public sector, that consume resources. The third, and probably most valuable, contribution concerns the economic evaluation of procedures designed to limit or prevent the spread of infection and would include inspection and regulatory activities.

National studies of *Salmonella* infection in Germany, Canada and England and Wales sought to evaluate, through the cost-benefit approach, which particular measure or combination of measures could achieve significant reductions in human salmonellosis. Because of the lack of data relating to some cost categories and reliance on expert opinion in some areas, the value of these studies was to provide a model for looking at costs and benefits rather than provide firm estimates of these. Nevertheless, estimations of the costs and expected success of a number of control measures indicated that, for example, irradiation of poultry meat and hygiene education aimed at both the domestic user and the food service sector would have benefit in reducing infection.

Most preventive activities are related to primary prevention aimed at preventing contaminated food reaching the general public, or secondary prevention aimed at identifying the cause of a problem and eliminating it; for example, when an outbreak of food poisoning occurs.

56.8.1 Primary Prevention

There currently is a need to bring together the critical evaluation of controls to reduce contamination in production processes with economic evaluation of those controls to estimate the potential benefits derived from them. Elimination of contamination early in the food chain would seem controllable and reliable, and therefore desirable, but its real effectiveness only could be calculated if the possibilities for recontamination and human illness were factored in. For example, the incidence of contamination of poultry carcasses exceeds that of the live birds, indicating that cross-contamination during processing is a significant factor in increasing risk of human infection.[50,57]

Even without detailed analyses, general statements on the benefit of a control procedure could be made. For example, although not a cost-benefit analysis, Levy and McIntyre[46] suggested that estimated outbreak costs of US $28,733 resulting from cross-contamination in a restaurant kitchen should be contrasted with the lower costs of primary preventive action such as hygiene education for restaurant food handlers ($500 per course attended by between 20 and 100 people), and regular inspection of the premises by public health officials (US $11 per visit; both at 1973 prices). In 1994, the United States Department of Agriculture (USDA) issued a ruling on mandatory safe handling statements on labeling of raw meat and poultry products.[35] These labels were intended to reduce foodborne illness by advising consumers on safe food handling practices, including thawing raw meat and poultry, cooking raw products, and treatment of leftovers. The cost of a pressure-sensitive label is between 1 and 2.5¢ with about $1,000 to redesign each label to be used in 253,000 food stores in the US. This compares with the cost of illnesses caused by meat and poultry contaminated by bacterial and parasitic pathogens (US $3,880 million to $4,330 million in 1992).[35] However, it would be extremely difficult to estimate the numbers of cases prevented by this approach to measure the actual benefits of the program.

Benefits of prevention probably would accrue over an extended period of time; therefore, any calculation would need to contain not only initial outlay and future maintenance costs, but also the future reduction in cases. On this basis, a measure that was not necessarily cost-beneficial in the first year might become so in time.[107] In 1995, the USDA developed a policy on pathogen reduction through HACCP and used as part of the proposal the economic benefits of HACCP to reduce foodborne disease costs.[36] The benefits accruing from introduction of HACCP systems in meat and poultry processing would be in the range of US $1.9 billion to $171.8 billion over 20 years (see Section 56.3).[23] The range was sensitive to the level of pathogen control achieved. Nevertheless, these benefits would be likely to exceed the costs of implementing and maintaining HACCP systems estimated to cost between US $1.1 and $1.3 billion over the same period.[23] In an alternative approach, the Swedish *Salmonella* control program for poultry, cattle, and swine, including testing animals and feed, sanitary slaughter, hygiene practice, and insurance, was evaluated at SEK 107.5 million in 1992 (Engvall et al., personal communication). This was about 10 times greater than the cost of domestically acquired illness; however, discontinuing the program could cost between SEK 115 to SEK 264 million annually. The success of a measure aimed at preventing, for example, salmonellosis, but that reduced other pathogens and other bacterial contamination would accrue additional benefits.

The desirable level of control or prevention is also a pertinent issue for consideration in developing or evaluating a program; thus eradication of infection in humans or animals may be either impracticable or prohibitively expensive.[32] However, reduction in human illness may be economically a more achievable goal. A German model has been presented where optimum combinations of measures could be expected to achieve a reduction in human illness of up to 65%.[44] This study attempted to identify measures or combinations of measures that would give a positive cost benefit. Given external factors, such as economic constraints on food production and customer tastes and requirements, this approach would allow a certain amount of freedom to choose not only the most cost-effective measures, but also those measures that are most acceptable to the producer or consumer. A Canadian study also took the approach that reduction of levels of poultryborne salmonellosis could be achieved by application of a number of measures.[24] It is necessary to identify those areas where preventive activities are likely to give the greatest benefit in terms of human illness prevented, the effectiveness of the procedure, and the return on investment. One way to achieve this is to measure the economic benefits that arise from specific interventions. Two interventions, with potential widespread application in the food industry and in poultry production, respectively based on postprocessing gamma irradiation to limit the level of contamination of the final product and competitive exclusion (CE) of salmonellosis during poultry production, are considered in Sections 56.9 and 56.10.

56.8.2 Secondary Prevention

The benefits of secondary prevention hardly have been considered at all and, although the scope for action is limited, the potential benefits may be large. These possibilities were explored in a study of a *Salmonella* outbreak attributed to contaminated cheese and in a study of a *Salmonella* outbreak in 1982 from contaminated chocolate.[20,64] The investigation costs of the latter outbreak at the point it was stopped (80% of product recalled) were compared with projected costs of the outbreak had it continued until stocks of the imported product were exhausted. The costs of illness associated with the 245 reported cases, of whom 51 were admitted to the hospital, were calculated to be £504,808 (1982 prices) for medical care, investigation, control, lost productivity, pain and suffering, and recall and destruction of product.[64] If the outbreak had continued until all the imported chocolate bars had been eaten, it was estimated that 36,000 cases would have occurred, 1,200 would have been reported, 230 people would have been hospitalized, and 5 people would have died. The potentially extended outbreak would have cost £1,673,826, over £1 million greater than the actual outbreak. Thus, for every £1 expended on investigation of the illness, there was a saving of £3.50 to the public sector, including outbreak control and medical treatment costs, and £1.50 in lost production. The greatest social and economic savings were for prevention of pain, suffering, and deaths. Benefits not only included the prevention of further illness in the outbreak, but also potential prevention of future outbreaks as a result of corrective measures made to the chocolate production process.

Although this type of economic study is unlikely to be repeated with many outbreak situations, it can be assumed that with a widely distributed contaminated product, intervention by companies and control agencies is justified economically as well as for public health reasons.

56.9 ESTIMATED COSTS AND BENEFITS OF FOOD IRRADIATION

Irradiation of specific foods has been considered by many developing and industrialized countries as an effective process to reduce spoilage and economic loss in harvested products; however, its potential for preventing foodborne illness has not yet been fully realized. Irradiation of specific food substances such as spices and irradiation of food for special "at risk" groups in the population is now common practice. For convenience and safety reasons, astronauts in extended flights have eaten irradiated ready-to-eat foods, and military personnel under field conditions plan to do so.[5,6,66] There also are medical reasons for other groups of people to consume foods with a reduced bacterial content. Hospitals, which provide food for a uniquely "at risk" population, including patients with cancer, acquired immune deficiency syndrome, and other conditions resulting in decreased immunity, are increasingly relying on wholesale suppliers to provide food for patients and staff. Commercial airline catering offers another possibility for provision of ready-to-eat foods that could be irradiated. Food poisoning incidents among airline passengers have been documented at least since 1947.[12,84] Foodborne disease is not a concern only for passengers, but also for crew, and has been considered a leading cause of pilot incapacity, with sufficient documented incidents to justify stringent standards for air-crew meals.[10] The possibility of irradiating the finished products before they are loaded into the aircraft provides an answer to this problem.[60] Other large-scale catering activities that could benefit from product irradiation include catering and centralized production of meals for schools and work canteens, etc. To be cost-effective, these would probably require a centralized large-scale operation with distribution of finished products to many locations. Scotland, England, Canada, and the US have examined the costs of poultry-related disease and the benefits of control procedures, including irradiation of carcasses; in 1997, the US Food and Drug Administration announced it was permitting irradiation of meats including poultry for human consumption.[33]

56.9.1 Poultry Irradiation in the UK

Because of the high capital costs of building and maintaining an irradiation plant and the need to factor in costs of the replacement and disposal of irradiation sources, the approach adopted for assessing benefits has been to calculate accruing benefits over a period of years. For example, a CBA study of poultry irradiation in England and Wales indicated that for 10 regional plants, irradiation of all poultry would have a positive cost benefit accruing over 15 years.[79] The costs of irradiation included the capital costs related to buildings, land, and equipment, and recurrent expenditure associated with overhead and operating expenses. These were modelled in an earlier study of a facility with a throughput of 380 tons per day at an average dose of 3 kGy.[107] Further allowance, in the study of poultry irradiation in England and Wales, was made for additional transport costs to the producer as well as an annual allowance for product promotion. A weighting of 25%, 50%, and 75% of the basic costs was added to incorporate any unforeseen plant and operating costs. The stream of benefits of irradiation (costs of salmonellosis less costs of irradiation) are presented in Table 56–9. These are presented as the net present values (1988 prices) discounted over 15 years, the life expectancy of an irradiation plant, using a weighted range of costs for a series of assumptions about the expected level of reduction in human salmonellosis at a discount value of 5%, the rate recommended by the UK Treasury for the appraisal of public works.[26]

The results indicated that only under the most severe assumptions about irradiation costs and effectiveness, for a 10% reduction in human salmonellosis overall, was the net benefit negative. Thus, benefit was negative only for the highest cost of irradiation (cost +75%) at the lowest assumption for cases avoided (10%). The flow of benefits increased several fold as reductions in human cases increased and were over £1.1 billion if total salmonellosis was reduced by 50%.

56.9.2 Poultry Irradiation in the US

Irradiation of poultry in the US was economically justifiable on the assumption that 81% of chicken carcasses could be irradiated practically, and that no one consuming these would contract campylobacteriosis, and only a few people would be infected with *Salmonella*.[66] However, costs did not include transportation of carcasses, promotion of the process, and removal of the used radioactive cobalt source although a high cost factor associated with deaths was included. These data were updated for the USDA's submission to allow irradiation of poultry in the US.[34] They concluded that the cost of irra-

Table 56–9 Flow of Benefits of Poultry Meat Irradiation Based on a Cost-Benefit Analysis for Irradiation of Poultry Meat in England and Wales over a 15-Year Period[79]

Costs of Irradiation	*Costs of Illness Avoided Minus Costs of Irradiation**		
	Reduction in Salmonellosis		
	10%	*25%*	*50%*
Basic cost	94.5	472.1	1,101.5
Cost +25%	60.6	438.3	1,067.7
Cost +50%	26.8	404.4	1,033.8
Cost +75%	−7.0	370.6	1,000.0

*£ millions (1988 prices).

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diation of 0.86¢ to 1.3¢/lb for treatment at 3 kGy would give a positive net public health benefit through savings in medical and productivity costs. This would be in the range of US $35 to $50 million (1991 prices) annually, even if only 10% of the carcasses were irradiated.

56.9.3 Poultry Irradiation in Canada

In Canada, one study showed that complete eradication of *Salmonella* from poultry was not cost-effective no matter what control measure was considered,[32] although another evaluation showed that limited control was more realistic.[25] Here, the costs of human salmonellosis were compared with various control procedures, and it was found that certain operations, such as cleaning crates, use of chlorine in chill water, educational programs, and irradiation were economical, although only the first and last of these were considered 100% effective in destroying *Salmonella* at those particular steps. Control measures also were costed over a 10-year period and annualized to compare with industry and society level costs. In terms of a strict test of effectiveness (90 to 100% reduction in human salmonellosis) and society benefits (US $17.5 million) over irradiation costs (US $17.1 million), irradiation was a viable control measure (costs updated to 1991 US dollars).[95] With fewer illnesses, costs to families and companies also would be reduced for a total benefit of US $28.6 million. The installation and operation costs over the 10-year period were US $14.5 million and $2.6 million for additional labor.

In all these studies, it is assumed that the consumer would pay the extra cost of plant decommissioning, but any obvious price differential between treated and untreated birds could be a discouragement to potential purchasers of the irradiated product. Because the state benefits from saved health costs, subsidies to companies choosing to use irradiation could keep the treated birds competitive in price.

56.9.4 Feed Irradiation

Another approach to reduce the presence of *Salmonella* in chickens was to use irradiated feed. A dose of 10 kGy irradiation increased the cost of by 3¢ per bird.[75] However, even if irradiated feed was the exclusive source of nutrient for broilers, *Salmonella* still would be present on many birds going to the slaughterhouse. Birds raised on irradiated feed probably would be more acceptable to retailers and consumers because no irradiation labeling would be required on the packaging, but less effective in reducing human illness.

56.10 COMPETITIVE EXCLUSION

An alternative approach to irradiation of poultry carcasses to reduce contamination with *Salmonella* would be the use of CE and antibiotics during breeding and rearing of poultry; thus birds would enter the processing plant with lower levels of contamination. This method probably would be less effective than irradiation but more acceptable to the consumer. In

Sweden, elimination of infected flocks, coupled with stringent sanitation procedures, use of pasteurized pelleted feed, and CE have been effective in keeping the infection rate in chickens low, but these measures are 5 times as expensive as the cleaning and disinfecting program used in the UK to control *Salmonella* contamination in poultry, resulting in birds that are more than twice as expensive for the consumer.[58,81,101]

Reducing the number of contaminated birds by CE, although unlikely to be as effective as irradiation, could have an important impact on the levels of human salmonellosis. Policies designed to reduce the numbers of infected birds in breeder flocks and the maintenance of high standards of hygiene at every level of production were credited with bringing about major reductions in numbers of contaminated birds and human infections in Sweden, Denmark, and Switzerland in the 1970s.[87] These policies, largely based on screening and destroying positive flocks and quarantine approaches, may be relatively expensive because of the potentially high level of wastage, particularly in the initial stages, and the need for adequate compensation for farmers to encourage compliance. Adoption of CE potentially could achieve a similar good result but avoid the wastage and cost.

56.10.1 Effectiveness of CE

CE was reported to have reduced *Salmonella* in flocks from 21% to 6.5% in Finland and 24% to 15% in the Netherlands.[81] In Canada, it was considered that CE treatment would reduce human salmonellosis costs associated with poultry by 41%; however, the costs to introduce the program would be greater than the benefits.[25] At 4.1¢/kg for a commercial CE product to be given to chicks throughout Canada, it would cost Can $26.2 million in contrast to the benefits to society, individuals, and industry of Can $22.84 million. Therefore, conflicting reports on the value of CE in reducing salmonellosis may reflect different control strategies in different countries and the acceptable costs per bird to the consumer.

56.10.2 Modelling of CE in England and Wales

In an evaluation of the benefits of a national CE policy in England and Wales, options for reducing *Salmonella* carriage in both breeder and broiler (chicken reared for the table) flocks were compared.[79] These were based on combinations of antibiotic and CE treatment of breeder and broiler flocks. Rather than attempt to estimate the levels of reduction of human *Salmonella* infection that could be achieved, for which little reliable information was available, the level of reduction above which these options would have a positive cost benefit in terms of the number of cases avoided was modelled. Thus, the cost benefits of reducing human infections in the range of 1% to 10% were considered although reductions in human infection as high as 40% or more may be possible.[24] The results of the option appraisal and the flow of benefits based on 1988 prices showed modest reductions of 1% to 5% in human infection could produce a positive net benefit depending on the combination of treatment options.

56.11 CONCLUDING REMARKS

Economic analysis has an important contribution to make in identifying where costs fall, and to the assessment of alternative preventive strategies. The WHO has recognized the importance of such studies and recommends that countries undertake assessment of the economic impact of foodborne disease.[104]

The resources available to invest in preventive activities are, however, likely to be limited. Thus, such activities should be targeted at the factors that have greatest influence, are amenable to modification, and that give the best possible return on investment. Considerable benefits could accrue from preventing poultryborne salmonellosis, and irradiation and CE could make an important contribution to reducing the economic and social effects of this disease. Although irradiation is expensive, its high level of efficiency in reducing contamination could result in a sharp decline in poultryborne salmonellosis if adopted on a large scale. Consumer opposition, however, could delay introduction for a long time to come. In contrast, CE offers a less effective but probably more acceptable alternative that would need to achieve only a modest reduction in human infection to give a positive return on its implementation. Consumers appear to be willing to pay more for safer food, although more research is needed in this area to delineate the conditions under which the shopper would purchase food safety. A study in the US showed that more than half of those interviewed would be willing to pay up to 17 ¢ per pound extra for chickens that are free of foodborne pathogens.[76]

The models presented could be adapted for comparing the relative benefits of alternative approaches to reducing contamination at one particular stage or

Table 56–10 Comparison of the Costs of Major Causes of Morbidity in the US

Disease	Cost (US $billion)*	Ranking
Diabetes 1	4–17.1	1–1
Heart disease	9.5	2–3
Hypertension	2.8–8.5	3–4
Obesity and related musculoskeletal disorders	1.9	4–7
Foodborne disease	0.2–5.5	5–5
Gallbladder disease	0.2–2.4	5–6
Cancer	0–16.5	7–2

* 1992 prices.

Source: Adapted with permission from N.D. Barnard, A. Nicholson, and J.L. Howard, The Medical Costs Attributable to Meat Consumption, *Preventative Medicine*, Vol. 24, pp. 646–655, © 1995, Academic Press, Inc.

of intervening at different stages in the production cycle. Such economic analysis of preventive strategies could provide a powerful adjunct to the HACCP approach, which seeks to identify and assess the microbiological hazards and risks associated with food production processes and identify points of control.[43,79] Economic analysis therefore would assist the process of deciding both, at which point intervention provides the greatest benefit and which alternative procedure would be most cost-effective at that point.

The relatively few published studies of the economic evaluation of foodborne disease suggest that the costs are high but that prevention can be economically worthwhile. Most authors indicate the figures they present are underestimates, often based on incomplete data and dependent on assumptions or expert opinion. To provide a more accurate assessment of the costs of foodborne disease, a number of methodological issues need to be addressed. These include the method of approach to costing foodborne illness, the relative contribution and valuation of intangible costs such as loss of life, estimation of levels of under-reporting, and allowance for different degrees of severity of illness. There also may be considerable costs associated with patients who develop long-term sequelae to their original infection. These may last from months to the rest of their life. Little information currently exists on the proportion of cases that develop such sequelae and whether they can be ascribed to a particular incident. Taking account of the numbers of reported and estimated unreported cases and those who develop long-term sequelae, the economic impact of foodborne disease is significant. This is further supported by an analysis of the major costs of meat-associated dietary and infectious disease in the US. Medical costs attributable to meat consumption ranged from US $0.2 to 2.4 billion for gallbladder disease to US $14 to $17.1 billion for diabetes, with foodborne disease, ranked fifth, responsible for US $0.2 to $5.5 billion (Table 56–10).[8]

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57

Transmissible Spongiform Encephalopathies

J. Gerald Collee

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References

Bovine spongiform encephalopathy, unfortunately known as "mad cow disease," was discovered in Britain in 1986. Fears that it may be transmissible to man have been discussed widely and considered in the food industry during the last decade. In this chapter, we set out the facts as we understand them up to 1998.

57.1 INTRODUCTION

Transmissible spongiform encephalopathies (TSE) are irreversibly fatal cerebral and cerebellar degenerative diseases affecting animals and man. Various forms are recognized presently over a fairly wide range of animal species (Table 57–1). The clinical presentation of the TSE is characteristic within each host species and the prominent features vary; signs include mental changes with altered temperament, apprehension and nervousness, incoordination with clumsy movements, tremors and unusual posture, changes in sensation and motor control with paresis, and an inexorably progressive and fatal outcome.[81,130] In some forms of the disease in sheep, there is itching (pruritus); the affected animal rubs itself against posts and buildings and the condition has been called "scrapie."

The neuropathological changes in these diseases also have many common features that confirm the diagnosis at autopsy. The hindbrain shows bilaterally symmetrical lesions with spherical or ovoid vacuoles in the grey matter that give stained sections a sponge-like appearance under the microscope (Figure 57–1).

Each of these diseases can be transmitted to another member of the same species by parenteral in-

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Table 57–1 Transmissible Spongiform Encephalopathies of Man and Animals[27]

Host Species	*Disease*	*Date of First Reports*
Humans	Kuru	1900
	Creutzfeldt-Jakob Disease (CJD)	1920
	CJD variant: Gerstmann-Sträussler-Scheinker syndrome (GSS)	1926
	CJD variant: Fatal familial insomnia (FFI)	1992
	"New variant" CJD (nvCJD)	1996
Sheep	Scrapie	1730
Goats	Scrapie	1872
Ranch-Bred Mink	Transmissible mink encephalopathy	1965
Mule Deer/Elk	Chronic wasting disease	1967
Cattle	Bovine spongiform encephalopathy (BSE)	1986
Captive Exotic Bovids: Nyala/ Gemsbok/Eland/Oryx/Kudu/ Bison/Ankole cow etc.	Exotic ungulate encephalopathy	1986-1989
Domestic Cats (and other Felidae in zoos)	Feline spongiform encephalopathy	1990

Source: Adapted with permission from J.G. Collee and R. Bradley, BSE: A Decade On—Parts 1 and 2, *Lancet*, Vol. 349, pp. 636–641, 715–721, © 1997, Lancet.

jection (eg, intracerebral injection) of homogenate of affected brain, hence the "transmissible" label. Transmissibility across species barriers is discussed later in this chapter.

The discovery of the first known cases of bovine spongiform encephalopathy (BSE) in British cattle in 1986[141] heralded the beginning of a catastrophic outbreak that rapidly caused national and international alarm. The consequences were of major economic importance, affecting farmers, animal breeders, meat producers, and pharmaceutical and other commercial interests related directly or indirectly to bovine products. The grounds for worry extended further into aspects of veterinary, dental, medical, and surgical practice;[142] for example, the source of a significant proportion of nonsynthetic so-called catgut sutures is bovine intestine, and fetal calf serum is widely used for the growth or stabilization of bacterial and viral preparations used in vaccines. Anxieties proliferated into the difficult area of the extensive use of bovine gelatin in confectionery and food production, and into other considerations of materials consumed or used by man that are not directly derived from cows but might have been associated with a bovine item during their production.[51,72,82,100–102,130]

There has been much speculation on the possible hazards of BSE to man, and the relevance of these considerations to food microbiology is clear.[24,52,65,100,126,133] Many informed biomedical and veterinary scientists considered it unlikely that the agent of BSE could be transmitted effectively to man by ingestion of naturally infected meat,[26,119,133,137,148] but there were clamantly expressed opposing views, and not the least of which were those of Professor Richard Lacey and Dr Dealler in England[39–41,43,91] and of Professor Diringer in Germany.[48] A linkage of BSE in cattle with the new variant form of Creutzfeldt-

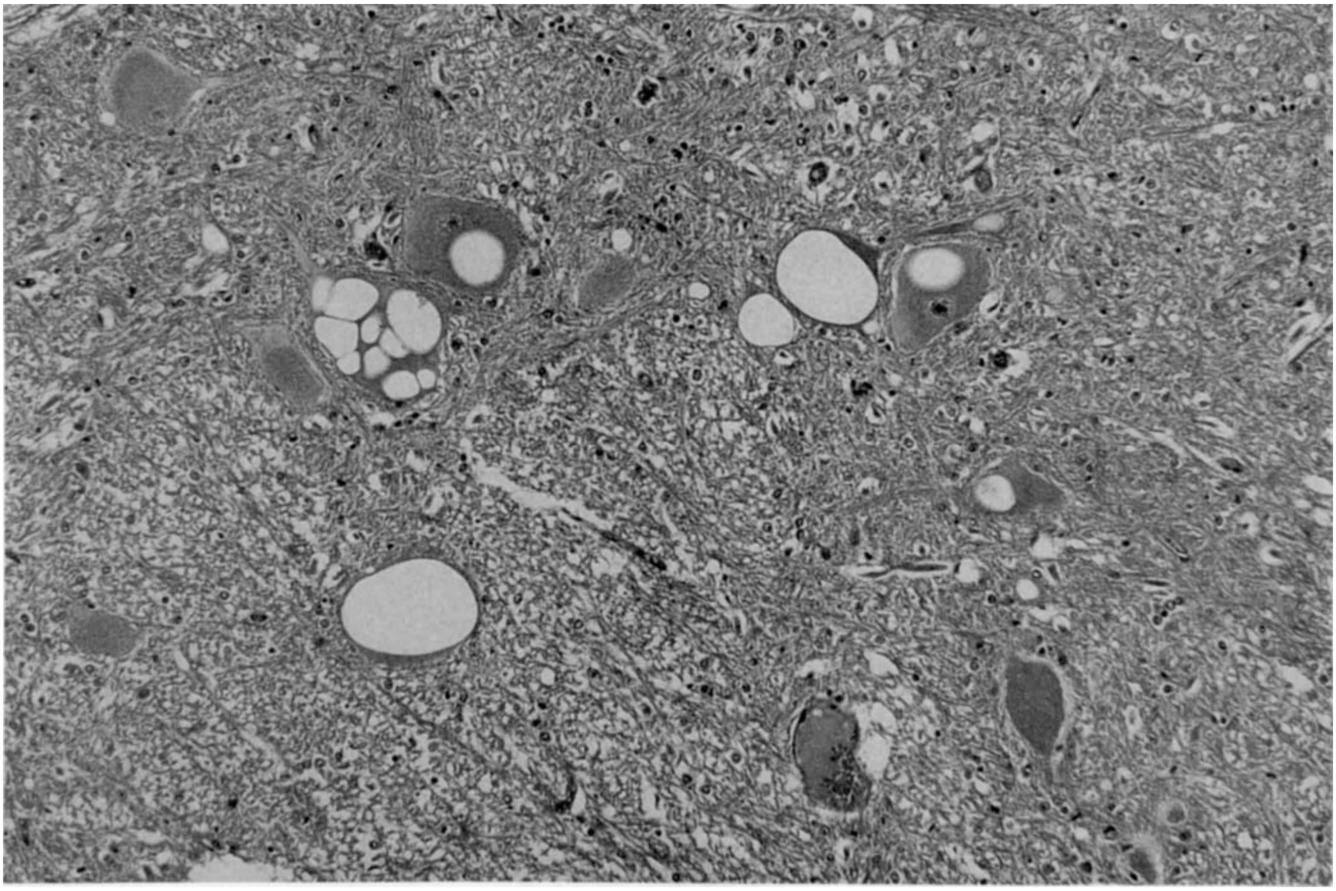

Figure 57–1 Neuronal Vacuolation and Spongiform Changes in the Brain of a Cow with BSE. Haematoxylin and eosin × 560*. Courtesy of S.A.C. Hawkins and R. Bradley.

Jakob disease (nvCJD) in man[150] was suspected[4,61,74] and is now confirmed.[5,20,68] The range of known spongiform encephalopathies of man and animals is considerable. Accordingly, this chapter will focus particularly on BSE in cows, the parallel model of scrapie in sheep, and the spongiform encephalopathies that affect man, notably CJD and kuru. The debated interrelationships of these diseases merit special consideration in the light of some possibly alarmist predictions. In view of the complexity of the arguments involved, some points are made more than once where it is important to establish their connection in this chapter.

57.2 BOVINE SPONGIFORM ENCEPHALOPATHY

Although BSE was first recognized in 1986, retrospective studies showed that some cases of the disease probably occurred in cattle in Britain early in 1985. The epidemic of this apparently new disease then developed progressively to require the destruction of many thousands of cattle in the next 10 years (Figure 57–2, Table 57–2). The data cited by different workers vary a little; this is because the definitive diagnosis of BSE takes some time. The figures given here have been corrected to reflect the numbers of confirmed cases that occurred in a given year rather than those for which notification was received in the year. Figure 57–2 shows how any commentary on the plot of cumulative data should be tempered with observations on the shape of the plot of annual numbers of new confirmed cases.

57.2.1 The Disease

A cow with BSE begins to behave strangely after a mean incubation period of 4 to 5 years from initial

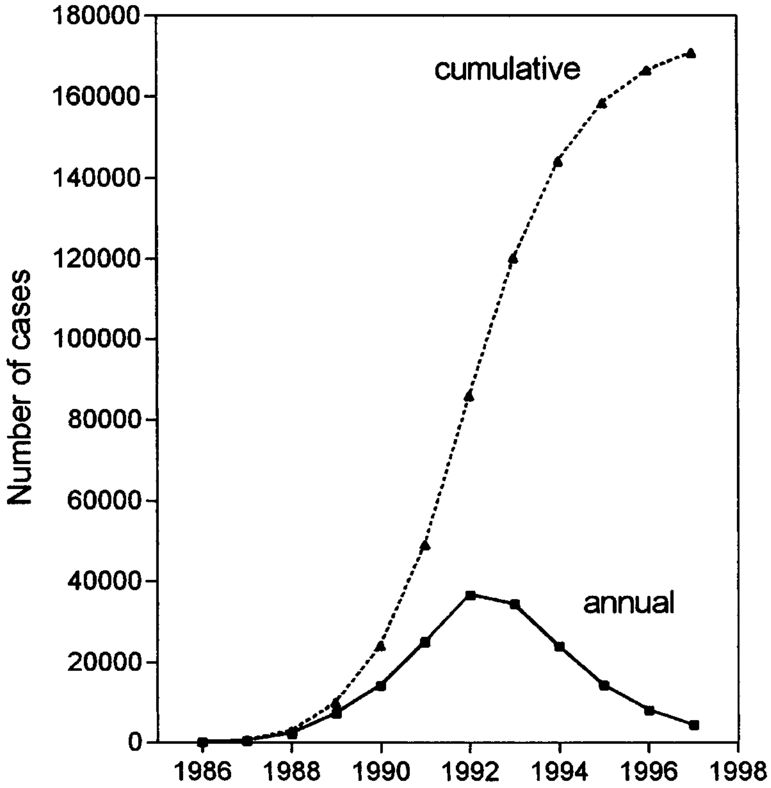

Figure 57–2 Annual Numbers and Cumulative Totals of Confirmed Cases of BSE in the UK, 1986–1997

infection. It licks its nose and grinds its teeth excessively as abnormalities of sensation develop. It becomes apprehensive and is unduly sensitive to touching or to noises. It is nervous when confronted and stands with its head low, its back arched, its feet splayed and stiff, sometimes swaying uncertainly with a wide-based stance and shying up suddenly and unsteadily. Its coordination deteriorates and it stumbles and slips. It is difficult to handle. It shows a fear of doorways that it cannot easily negotiate. It may show cutaneous shivering or trembling of its limbs, and it may become frenzied. It loses condition, appetite, and weight, and reduces its milk production. The duration of the distressing disease is usually a few months from its initial insidious presentation, but the period of illness ranges from less than a month to more than a year until its invariably fatal outcome.

57.2.2 Early Investigations

Very intensive neuropathological and epidemiological studies were set up promptly in the mid-1980s and thereafter by the British authorities responsible for veterinary surveillance.[13,32,45,101,102,130,137,144–147] The early clinicopathological findings indicated striking parallels between BSE in cattle and scrapie in sheep, and epidemiological investigations pointed to proprietary feedstuffs as the likely vehicle of infection of BSE in cows. Subsequent evidence incriminated meat and bone meal (MBM) concentrate in animal feed as the source of the disease. MBM is obtained by the process of "rendering" discarded animal and poultry carcasses and other animal products collected for recovery of their commercially useful constituents (see below). The numbers of sheep in Britain had increased in the 1970s, and scrapie is an endemic disease of sheep in Britain (and many other countries). Hence, the ultimate sources of BSE were assumed to be infected carcasses and offals of sheep carrying the agent of scrapie and, on recycling through the rendering process, offals of cattle infected with the agent. BSE was deemed to be scrapie in cows. This implies that scrapie, long held to be a disease of sheep and goats but not of cows, had somehow broken through a natural "species barrier" that had protected cows for more

Table 57–2 The Annual Occurrence of BSE in British Cattle from 1986*

Year	*Annual Numbers*	*Cumulative Numbers*
1986	63	63
1987	473	536
1988*	2184	2720
1989	7137	9857
1990	14181	24038
1991	25032	49070
1992	36682	85752
1993	34370	120122
1994	23944	144066
1995	14300	158366
1996	8016	166382
1997	4312	170682

Up to 26 June, 1998, 171,935 cases have been confirmed on 34,522 farms (confirmed data to mid-1998).

**BSE was not made notifiable until June 1988.*

Source: Data from J. Wilesmith and R. Bradley, Central Veterinary Laboratory, UK, and D. Matthews, MAFF, Tolworth.

than 2 centuries before the events of the 1980s. An alternative possibility, that BSE is primarily a disease of cattle and was unrecognized over the years until the discovery of BSE in 1986, continues to merit consideration. This is discussed later in this chapter.

The initial epidemiological studies[147] revealed that the incubation period for the disease ranged from 2 to 8 years. The modal age of occurrence (and incubation period) is 5 years. The full age range of disease occurrence is now recorded as 20 months to 18 years. Most of the cases were assumed to have been infected as calves. Female cattle have been much more commonly affected than male animals. BSE predominantly hit dairy herds; the disease was much less frequently seen in beef suckler herds. This was attributed to the fact that protein feed supplements are much more commonly used in the management of dairy herds. After sucking colostrum, dairy calves are removed from their mothers and given milk substitute and then weaned on hay and protein supplement. Beef breed calves are suckled with minimum feed supplementation. Indeed, some female cross-bred animals born in dairy herds and taken into beef suckler herds could well have been infected before their transfer and thus account for a significant proportion of BSE cases in suckler herds. The lifetime of a male calf may be relatively short and the numbers of bulls that are allowed to reach maturity are relatively small because artificial insemination is commercially exploited. There are therefore several reasons why the expressed disease was seen predominantly in female cattle in dairy herds.

In general, only one or two animals in any one herd went down with the disease; the occurrence of more than 3 or 4 cases in a herd (mean size 80 animals) was unusual, but some herds were very severely affected. At the beginning of the outbreak, index cases seemed to occur at about the same time at many different farms throughout Britain. As the outbreak developed, the incidence of BSE was highest in farms in the south and east of England and lowest in Scotland, with no early cases in Ireland. The initial pattern of occurrence of the disease suggested that there was a common source that was widely distributed, that we were observing an "extended common source epidemic," and there was little doubt that the infective challenge had been delivered in the ruminant-derived protein feed supplements supplied from rendering plants. The possibility that subtoxic levels of organo-phosphorus compounds used in farming are involved as direct factors or indirect cofactors in BSE has been largely discounted, but still is being researched.

The likely period when the cows first were challenged with the agent of BSE in this way was the winter of 1981–1982. It is important to appreciate the range and the detailed nature of the early studies instituted immediately after the disease was recognized in 1986. By December 1987, findings based on thorough investigation of the first 200 affected herds were as follows.

1. BSE is not simply genetic in origin. A genetic predisposition in cattle has been considered,[143] but no breed disposition has been noted among the cattle affected by BSE.[76,130,145,146]
2. BSE is not attributable to a recognized chemical toxin.
3. BSE is not associated with the use of an identified vaccine or other commercial biological preparation.

In the latter part of 1988, it was shown by Fraser et al. that BSE is transmissible to mice.[55]

A working party on BSE, set up in 1988 under the chairmanship of Professor Sir Richard Southwood, produced the Southwood Report in February 1989.[119] The British government already had made BSE a notifiable disease (June 1988) and, in the next month, introduced a temporary ban on the supply of ruminant-derived protein for ruminant feed supplements following studies by Wilesmith published in 1988.[147] The Southwood Committee then recommended in-definite continuation of the ban. Further studies by Wilesmith and Bradley and their collaborators were published in 1991–1993.[13,144–147]

57.2.3 The BSE Agent

The transmissible agent has not been visualized definitively and does not have the characteristics of a bacterium or a conventional virus. In its pathogenesis and in its resistance (in preparations of homogenates of infected brain) to chemicals, heat, and radiation, it behaves like the scrapie agent and these features are described in more detail below.

57.2.4 A Note on Rendering

Animal carcasses and waste animal material are rendered into commercially useful MBM by various processes that include heating to remove water and to liberate fat (Figure 57–3). A detailed survey of all 46 rendering plants in the United Kingdom (UK) by Wilesmith and his colleagues[145] in 1988 is very informative. The raw material included fat trimmings, bones, offal, whole carcasses, and other mixed material. The sources were bovine, ovine, porcine, poultry, and others. The initial products are liquid fats (tallow) and the separated protein-rich residual solids are called greaves; the latter can be further processed to yield MBM. Most of the plants used batch processing, but 13 plants had more modern continuous systems. Essentially, the raw materials are broken down mechanically to an acceptable particle size (ranging from 1 cm to 10 cm) and then heat treated at temperatures ranging from less than 100° C to 150° C. The periods of exposure to the maximum temperature for each of the processes studied varied widely.

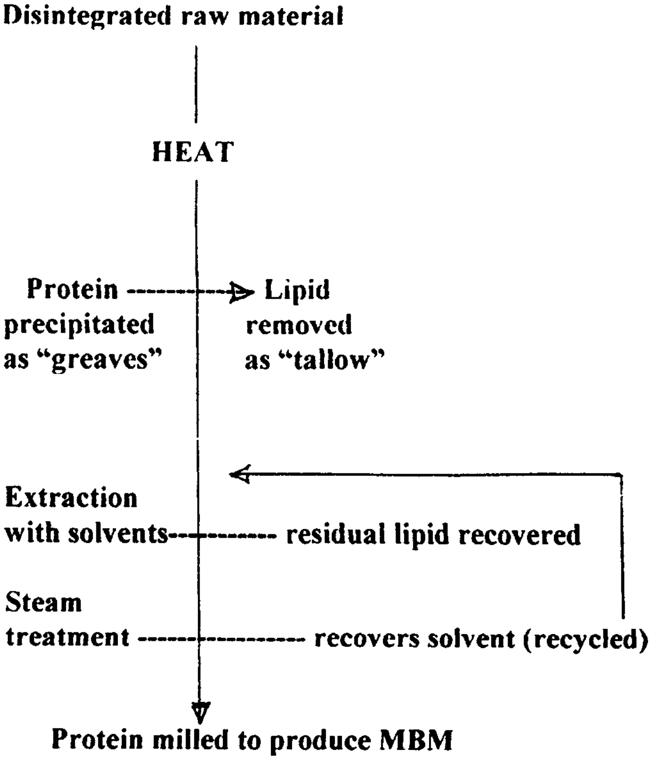
Disintegrated raw material
HEAT
Protein precipitated as "greaves"
Lipid removed as "tallow"
Extraction with solvents
residual lipid recovered
Steam treatment
recovers solvent (recycled)
Protein milled to produce MBM

Figure 57–3 An Outline of the Rendering Process

An important change had occurred in the late 1970s and early 1980s. The processing of greaves traditionally had included a hydrocarbon solvent treatment at about 70° C for 8 hours to increase the extraction of tallow by treatment with hydrocarbon solvents such as benzene, petroleum, hexane, perchlorethylene, or trichlorethylene. The protein meal produced in this way had a very low fat content. Residual solvent finally was removed from the solid product by heating and by exposure to pressurized steam (live steam stripping). As the market for tallow declined and, simultaneously, as the fire and explosion hazards of the hydrocarbon extraction processes were recognized and safety measures were more stringently enforced, the hydrocarbon solvent treatment and extraction steps were omitted by many processing plants. Thus, the fat content of the MBM produced by these plants, or by processors using their material, was increased significantly. The protective effect of fat for microorganisms is well known. When the solvent extraction procedure was used, it may have assisted decontamination because the second (solvent recovery) heat step took place in a relatively low fat environment. At the time of the 1988 survey, the only two plants using solvent extraction were in Scotland, and they produced most of the MBM made in Scotland in 1988. The rapid reduction in the use of the solvent step by English producers during the period 1977 to 1982, and hence the great increase in the proportion of unextracted MBM made available for use as cattle feed supplements, is dramatically evident in the data produced by the Wilesmith team.[145] Bear in mind that it is thought that the initial BSE challenges were delivered to British cattle in 1981–1982. If the exclusion of the solvent step (and the loss of the additional heat-treatment involved in solvent removal) had nothing to do with the apparent boost in the challenges assumed to have been delivered in the late 1970s and early 1980s, this must be a remarkable coincidence. However, some reviewers indicate reservations and suggest that changes in rendering procedures began much earlier—in the early 1960s.

57.3 SCRAPIE

This fatal cerebral degenerative disease of sheep and goats was recognized in western Europe in the early 18th century. Countries in which the disease has occurred in sheep in the last two centuries include England and Wales, Scotland, Spain, Germany, Hungary, France, Italy, Switzerland, The Netherlands, Iceland, Cyprus, and Norway.[130] In some of these countries, the disease is endemic (enzootic). In others, it has arisen in imported sheep. It has been kept out of Australia and New Zealand by a policy of quarantine followed by compulsory slaughter of affected imported sheep and flockmates, with due compensation. Scrapie has also occurred in North America, southern Africa, India, and Japan. Countries now apparently free from scrapie include Australia, New Zealand, Denmark, Argentina, and Uruguay.[130]

57.3.1 The Disease

Scrapie most commonly affects breeding ewes aged 2 to 4 years and presents as loss of coordination affecting the hind limbs. Disordered sensation commonly, but not invariably, gives rise to itching. The animals then scrape themselves against walls, gates, posts, and trees so that there is patchy loss of wool with lesions developing in the underlying skin. Scrapie follows an inexorably fatal course; affected sheep gradually go down and die after several weeks or months. The histopathology of scrapie is that of a spongiform encephalopathy. There is no inflammatory cell reaction and there is no antibody response.

Scrapie is transmissible to sheep by parenteral injection of infected brain. The incubation period varies from about 5 months to 5 years, depending on the strain of scrapie agent concerned and the genetics of the recipient sheep. The influence of the host genotype on the course of the disease is of special interest. The disease in sheep (and in goats) can be transmitted to mice, but this is not invariably successful. Intensive studies of scrapie in mice have yielded much valuable information, including the finding that more than 20 different strains of the scrapie agent can be characterized and the fact that host genetic control of the experimental disease can be demonstrated with different strains of mice.[46,81,82]

57.3.2 The Scrapie Agent

When it was shown in 1936 that scrapie is a transmissible infection, scientists began a long and difficult search for the elusive causative organism. It became clear that this was not a metabolic, chemical, parasitic, protozoal, or bacterial disease and, in due course, scrapie was assumed to be a viral infection. In view of its long incubation period in sheep, it was regarded as a "slow infection"—a term introduced by Sigurdsson to embrace various diseases that typically had long incubation periods. The evolution of the concept and the development of the notion of the unconventional agent of scrapie, because its nature and debated taxonomy continued to puzzle investigators, is brilliantly described by Kimberlin.[81]

The terms *viroid*, *virino*, and *prion* were coined successively to take account of evolving views, and the validity of these terms was contested fiercely. In late 1998, the scrapie agent are generally regarded as a prion as originally conceived by Stanley Prusiner in 1982.[112–115] A prion is a proteinaceous infectious particle apparently devoid of nucleic acid. Prions, as defined, are detectable in the high infectivity fraction of brain homogenate from mice experimentally infected with scrapie from sheep. Prion theory is still debated.[22,54,95]

Why is the scrapie agent not thought to be a bacterium or a conventional virus? The infectivity of affected brain homogenate passes through bacteriological filters of very small pore size (eg, 50 *n*m and perhaps much smaller). It is unusually resistant to destruction by heat, by antimicrobial chemicals, and by ultraviolet or ionizing radiation. For example, the infectivity of brain homogenate is not lost after it has been boiled in water and it is not assuredly destroyed by standard sterilizing exposures to wet heat, such as autoclaving at 121° C for 15 minutes; exposure to 134° C to 138° C for 18 minutes in a porous load cycle may achieve inactivation, but this sometimes is inadequate. It is partially inactivated by phenol but not by alcohol or by strong disinfectants such as formaldehyde or glutaraldehyde. Exposure to 2% available chlorine as sodium hypochlorite for 1 hour may be effective, but sodium hydroxide is not assuredly inactivating.[135,136]

No causative organism can be visualized in stained preparations under the light microscope. The agent has apparently not been visualized by electron microscopy (EM); however, in negatively stained EM preparations of detergent extracts of affected brain treated with protease (proteinase K), abnormal scrapie-associated fibrils (SAF) occur. These are long strands of material composed of an abnormal form of host glycoprotein called prion protein (PrP). The normal (cellular) form is designated PrP^C and is present in normal brain cells and other neurones; the modified form in scrapie is designated PrP^{Sc}. The latter is resistant to digestion by proteinase and is not altered by treatment with enzymes that hydrolyze or modify nucleic acids. PrP^C can be digested by proteinase K, but the relatively resistant scrapie isoform PrP^{Sc} undergoes limited hydrolysis of the N-terminal amino acids to yield a product referred to as PrP27–30. The term PrP^{res} has been introduced as a general term for such abnormal (resistant) PrP. According to Prusiner, a prion rod (SAF) is an aggregate of prions composed virtually entirely of PrP27–30 molecules. Thus, detergent extraction and limited proteolysis of scrapie-infected brain containing PrP^{Sc} yields fibrils visible by negative staining in the electron microscope. These can be studied also in scrapie-infected spleens[122] or in central nervous tissue from cows with BSE (Figure 57–4). When SAF are purified, there is some copurification of infectivity so that the infectivity titer is increased, and it is possible that scrapie-associated fibrils of modified PrP are indeed the infectious agent. Bear in mind, however, that experienced laboratory workers describe the agent of scrapie as very sticky; accordingly, it might stick to an aggregate and copurify with it.[122,128] Moreover, opinions on SAF are divided and some workers hold that these may be artifacts produced when the infected material (such as brain or spleen) is processed for EM. Abnormal PrP is not seen as fibrils in stained sections of infected tissue, but one must allow for the greater magnification and resolution of the electron microscope. The abnormal PrP that can be visualized by immunostaining is not directly correlated with infectivity, and PrP^{res} with apparently no infectivity can be generated.[3]

The transmissible agent of scrapie has been cultured in some experimental studies but has not been cultured effectively in any system. It can be passed experimentally from sheep to sheep and goats,[109] from goats to mice, and from sheep to mice and hamsters, causing typical patterns of disease in these animals. It can be passed also to rats and cattle.[57,130] Many biologically different strains are characterized by the pathogenesis of the disease observed in mice. Some of the strains can mutate. In this context, Kimberlin has posed the critical questions: If the scrapie agent is a virus or a virino, we need to find the scrapie-specific nucleic acid that has eluded us so far. If scrapie is caused by a prion devoid of nucleic acid, and if prion theory holds sway, how do we account for the observed scrapie strain variation and mutation in sheep and in experimentally infected mice? Some microbiologists still find it difficult to accept that this can be achieved without a nucleic acid component. For example, Asher[8] and the Addendum to Chapter 6 of the Spongiform Encephalopathy Advisory Committee's Report of 1994[130] indicate reservations that are held. Some virologists maintain that nucleic acid would be detectable by more modern methods, but they have not produced proof of their view so far. Alper[6] brought biophysical evidence to support the view that the agent is devoid of nucleic acid and is derived from domains of nerve cell plasma membrane lacking normal PrP molecules. The debate continues and this was reviewed by Manuelidis in 1997.[97]

It is not absolutely clear that there is direct vertical (ie, transplacental) transmission of scrapie, but there is a fair amount of indirect evidence suggesting that prenatal or perinatal infection can occur. The maternal transmission of scrapie in sheep, from ewe to lamb, is said to be common but is attributed largely to infection of the lamb at birth from contaminated placenta. The latter also accounts for lateral transmission to other sheep by consumption of placenta (by adult sheep[109]) or by pasture contamination. The survival and persistence of infective prions in soil have been demonstrated. It seems that mites harbor scrapie prions in affected pastures,[158] but the significance of this is not clear. The incidence of scrapie in the offspring of the flock at lambing time increases in proportion to the time that lambs are held and allowed to run with their ewes.[81] Ridley and Baker[121] questioned the whole concept of maternal transmission of spongiform encephalopathy, but there is now evidence suggesting that some maternal or perinatal transmission of BSE occurs at a relatively low level,[7] say 10% under experimental conditions and perhaps about 1% under field conditions. This still is debated.

Much work done by Dickinson and his team at Edinburgh has afforded insight into the complex genetics of host susceptibility to scrapie in sheep[46] and to strain variation and mechanisms of pathogenicity of the scrapie agent.[47] The pioneering work was developed further by Kimberlin and colleagues.[84] The length of the incubation period of scrapie is dictated by the aggressiveness of the strain of infecting agent and the genotype of the host. The *Sip* gene (scrapie incubation period gene, with two alleles sA and pA) influences resistance and hence the incubation period in sheep. Homozygotes for sA are the most susceptible to natural scrapie, and sheep homozygous

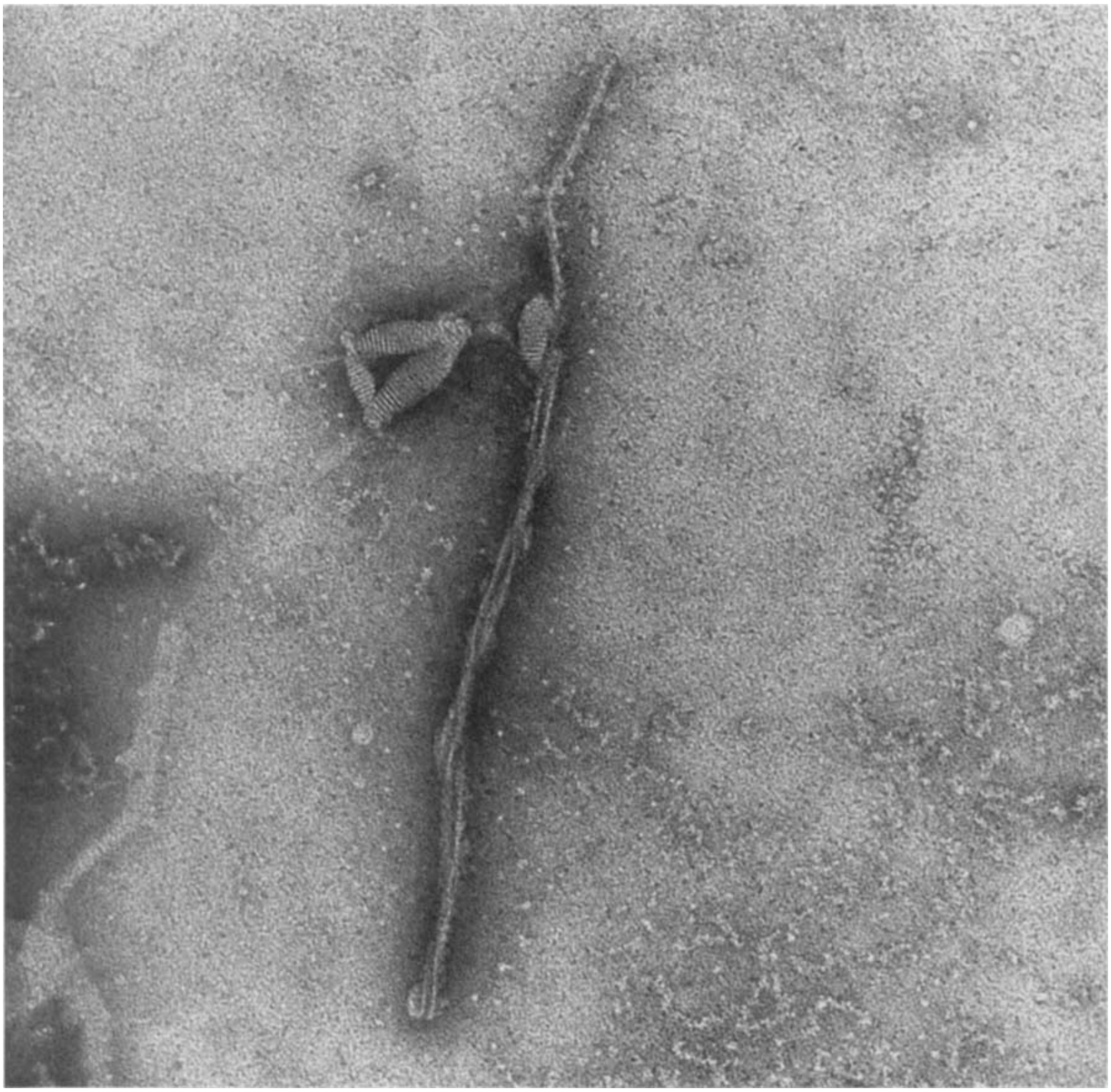

Figure 57–4 BSE Fibrils in a Preparation of Bovine Spinal Cord Tissue. N-lauroylsarcosine detergent extraction and proteinase K digestion followed by negative contrast transmission electron microscopy. EM × 125,000. Courtesy of M. Stack and R. Bradley.

for pA have been shown to be much less susceptible to experimental scrapie. The *Sip* gene in sheep is probably the same as the *PrP* gene (discussed elsewhere in this chapter). Paradoxically, sApA heterozygotes are susceptible to experimental scrapie but apparently not susceptible to natural scrapie. These considerations have practical value in developing relatively resistant flocks, but a word of caution should be added. If the *Sip* gene in sheep acts as the analogous *Sinc* gene in mice, Kimberlin points out that ewes of a resistant *Sip* genotype could be healthy carriers of disease and a potential source of danger to their lambs and other sheep of a susceptible genotype. Similarly, a *Sip* mutation in the host animal could probably derepress the susceptibility mechanism (or switch off the resistance mechanism) to allow disease to be expressed; and a mutation in the carried noninvasive strain of scrapie could change it to a neuroinvasive strain.[81–83] These are speculative theories that merit continuing consideration in relation to various models of disease presented by the spongiform encephalopathies.

57.4 TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES IN OTHER ANIMALS

TSE of commercially reared (ranch-bred) mink, transmissible mink encephalopathy (TME), was first recognized in Wisconsin in 1947 and has been attributed to the feeding of scrapie-contaminated tissue.[21,66] The high mortality of TME is notable in some of the outbreaks, with no apparent secondary spread unless cannibalism occurs. Infection is assumed to have been solely by ingestion, although bite wounds and scratches are common in these animals; bites sustained when feeding could be a more effective route.[133] The ingestion of untreated abattoir waste and dead stock possibly contaminated with scrapie prions in mink feed is likely to be the factor associated with high challenge doses of the infecting agent.[21,82]

In the late 1970s and early 1980s, a chronic wasting disease of captive mule deer and elk, with the features of a TSE, was reported in the United States (US).[154,155] The affected animals either had been reared or held for some years in captivity, but more recent findings indicate that cases of chronic wasting disease have occurred in free-ranging elk and deer in the US and that lateral transmission may occur by direct contact or as a result of environmental contamination.[156]

BSE-like disease has occurred during the last decade in 19 captive exotic ruminants of seven different species in Britain up to mid-1998[86,87,130] (Ray Bradley and Dr. D. Matthews, personal communications). The source of the infection in the early cases has been assumed to be the ruminant-derived protein feed supplements that caused the outbreak in cattle. Some of the affected exotic ruminants were born after the ruminant feed ban. It seems that some exotic species are particularly susceptible and that lateral and possibly maternal transmission of the disease may have occurred in some cases, but it is also possible that infected feed was fed unknowingly to them, as it was to some cattle, after the ban.

In 1990, spongiform encephalopathy was discovered in domestic cats in Britain. By mid-1998, 85 cases of the feline disease (FSE) had been confirmed in British mainland cats. Most of these cats were aged 5 to 6 years when affected. The annual numbers of cases from 1990 to 1997 were 16, 11, 13, 10, 14, 8, 6, and 6. Only one cat with FSE was born after the extension of the offal feed ban to include pet foods; the evidence points to a feed source, with BSE rather than scrapie as the most likely origin. FSE was reported in a cat in Norway in 1995. There has been one case also in Northern Ireland and one in Liechtenstein. The agent of FSE has now been shown to be identical with that of BSE.[20] FSE also was reported and confirmed in several captive large cats (nine cheetahs, three pumas, one tiger, and two ocelots at mid-1998) born in British zoos. These animals had been fed raw cattle carcasses and had eaten infected brain or central nervous tissue. In 1990, the experimental transmission of BSE to pigs was reported.[38] Transmission was achieved by parenteral inoculation of very large doses; this model bears no resemblance to any likely natural challenge. The disease has not been transmitted to pigs following challenge by feeding; this study is not yet quite complete, but it is now longer than 8 years postchallenge.

57.5 MODELS OF HUMAN PRION DISEASES

Despite the world's long experience of scrapie in sheep, there is no evidence that the scrapie agent has caused disease in man. Why, then, should we be considering scrapie and other animal models in such detail in a chapter concerning the possible risk of foodborne BSE to man? The reason is that there are many parallels between scrapie in sheep and BSE in cows, and there are now debated connections between these diseases and the known spongiform encephalopathies of man that include kuru (a disease affecting the Fore tribe in Papua, New Guinea) and CJD. Very substantial evidence reviewed by Kimberlin and others[81] indicated that CJD and its previously known variants up to 1995 were not epidemiologically linked to scrapie. Thus, although BSE may be a form of bovine scrapie, CJD in man did not seem to be human scrapie—unless a theory proposed by Diringer[48] gains currency.

57.5.1 Kuru

The investigation of an unusual disease occasionally provides clues to mechanisms of wide biomedical interest. So it was with this disease that affected the people of the South Fore highlands in Papua, New Guinea. The story is told by Gajdusek and Zigas,[56] who traveled to the Fore region in 1955. At that time and into the 1960s, kuru accounted for many deaths, predominantly of women and young children, among the South Fore natives.

Early signs of the disease included unsteadiness and poor coordination with clumsy movements; there was cerebral and especially cerebellar dysfunction. The course of the disease ranged from several months to 1 to 2 years in adults, and it progressed more rapidly in children. It was invariably fatal and virtually took middle-aged and older women out of the population.[56,114]

The infection was linked to the native practice of ritual cannibalism associated with mourning ceremonies for relatives within the tribe (endocannibalism). The distribution of the disease was attributed to the fact that women and young children ate the brains and offal, whereas the men ate the muscle. Whether the agent of kuru might be transmitted by routes other than by ingestion—such as contamination of the eyes or abraded skin or mucous membranes—is still debated because part of the mourning ceremony included smearing remains on the bodies of the mourners.

Gajdusek suggested that the original source of infection may have been a sporadic case of CJD that died in the tribe at the turn of the century and was subjected to the ritual; the infection then would have been recycled progressively through the relatively small population of the South Fore people. The neuropathology is that of a typical spongiform encephalopathy with degenerative vacuolar lesions in the brain and cerebellum and with no inflammatory reaction, but with PrP-positive and amyloid-positive kuru plaques. Cannibalism was stopped in the mid-1950s and was followed by a clear reduction in cases among younger children and a steady advance in the age of onset of the disease as the slow fuse burned out in the community. The long incubation period of 20 to 40 or more years for adults accounts for cases occurring into the present decade, but no South Fore native born after 1959 has developed kuru. The disease is disappearing.[114]

It seems that a direct route of infection was essential for the expression of kuru, with no secondary cases. Studies of the disease in children and mothers have shown no evidence of prenatal, perinatal, or neonatal infection transmitted from mother to child; thus, transplacental infection or infection by ingestion of human milk does not seem to have occurred.

Kuru is transmissible by intracerebral inoculation to apes and monkeys. Early attempts at per-oral transmission failed, most probably because of inadequate dosage, but also perhaps because intragastric intubation was used (Dr C.J. Gibbs, personal communication). Successful oral transmission to monkeys with primate-passaged brain was achieved later.[58]

57.5.2 Creutzfeldt-Jakob Disease

This is a spongiform encephalopathy of man with a worldwide distribution and an incidence of about 1 in 1 to 2 million people annually. Some known clusters of cases, for example in Sephardic Jews of Libyan descent and in a relatively inbred community in Slovakia, are attributed to a genetically linked susceptibility.

There are three known forms of CJD.

1. ***Sporadic CJD*** is presumably caused by a spontaneous somatic mutation in the *PrP* gene with the production in the host of the abnormal form of PrP, or by a chance transformation of the normal protein to an abnormal protease-resistant self-replicating form, or it may be the result of a mutation in a silent (latent carried) strain of the CJD agent that changes to neurovirulence. Sporadic CJD accounts for about 85% of cases.
2. ***Inherited*** or familial (genetically determined) CJD is associated with germ-line mutations of varying penetrance that predispose the person to CJD. This accounts for 12% to 15% of cases.
3. ***Iatrogenic CJD*** results from the injection or implantation of hormones or tissue, or a surgical device contaminated with material from a human case or carrier of CJD. Examples of iatrogenic infection include cases attributed to contaminated electroencephalogram electrode implantation, contaminated neurosurgical instruments, implantation of infected dura mater, a corneal transplantation, and injections of human growth hormone or human gonadotrophin derived from human pituitary glands. The accounts of the iatrogenic transmission of the CJD agent are well established in the literature and have been reviewed comprehensively by Prusiner.[114]

Note: Previously recognized clinical variants of CJD occur as the Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia. These are not further discussed in this chapter.

It has been difficult to accept at face value the observations of sporadic CJD on the possible development of endogenous CJD by mutation of a host gene (see, for example, discussion by Johnson[79]), for this challenges our accepted dogma in relation to infection. If a sporadic form of a potentially infectious disease can arise de novo in a new host, we would have to accept that Needham's long-debunked views on spontaneous generation merit rehabilitation. Kimberlin suggests a way out.[81] He theorizes that CJD may exist in latent silent form as a widespread infection of man and that this is maintained in the population, largely without expression of the disease. On rare occasions, there might be a spontaneous mutation to neurovirulence in an unfortunate host and this would ultimately give rise to a sporadic case of CJD. Perhaps undue genetically determined susceptibility would account for the higher frequency of familial cases in this model. At present, we lack sufficiently sensitive detection methods for CJD to test this concept of the occurrence of pathogenic and nonpathogenic or host-repressed strains. The postmortem diagnosis of CJD rests on the histopathological evidence and on the identification of abnormal PrP^{Sc} (or PrP^{CJD}) by partial purification of infected brain homogenate and extraction to yield material for an immunoblotting procedure with anti-PrP sera, by a dot blot procedure, or a histoblotting technique after prior proteinase digestion and chemical denaturation.

Prusiner has considered the molecular genetics of inherited human prion diseases with much care. He paraphrases the Kimberlin theory of a putative latent agent in a worldwide reservoir of humans and links this with the views of Chesebro on PrP and the scrapie agent.[22] Thus, he seemed to be driving us to the view that there may be a common prion lying latent in most of us. The development of CJD is a rare, random event (excluding the iatrogenic routes), whereas almost everyone with *PrP* point mutations or inserts appears eventually to develop neurological dysfunction. An exception is mutation at codon 200, which has only 50% penetrance. Prusiner referred ominously to a worldwide pool of potentially dangerous prions that may occur in humans, animals, and insects without causing detectable illness. He was moderately reassuring about possible foodborne challenges, making the point then that there was no evidence to support the hypothesis that sporadic CJD could be attributed to prions being ubiquitous in our food chain; and he observed that infection by the oral route is extremely inefficient in the hamster model. He did not dismiss the possibility that CJD may arise spontaneously and endogenously without any exogenous prion source, and he considered that somatic mutation of the *PrP* gene is a likely mechanism. He did not favor the notion of a ubiquitous virus that is somehow switched on when the *PrP* gene codes for a mutant protein that facilitates the expression of CJD infection. In Prusiner's view, the prion can achieve all of this, given the right circumstances, without the help of viral nucleic acid. As the prion diseases stand apart from conventional virology, Prusiner asked us to consider them as pseudoinfections that do not obey rules that we have previously laid down in our textbooks.

The resistance of the transmissible agent of CJD is comparable with that of the scrapie agent. Accordingly, special precautions must be taken in dealing with tissues that might contain the CJD agent and in the subsequent use or disposal of any potentially contaminated instruments. In the UK, the Advisory Committee on Dangerous Pathogens has issued guidance on precautions that should be observed.[1] It is of interest that Dr D.W. Burdon of Birmingham, England, studied a very resistant agent occurring in human ileal fluid that he named ileal-fluid dependent organism (IFDO) in the 1980s.[50] This had some properties in common with CJD agent, but there are differences.[129] Whether the IFDO agent is an organism in the accepted sense or a replicating crystalline form derived from the medium is uncertain. Awareness of such a phenomenon is important at a time when there is a strenuous search for prion-like agents in human tissues. Meanwhile, Collinge and his colleagues[29,30] have pointed out that screening of selected patients by *PrP* gene analysis may reveal that sporadic CJD has been underdiagnosed by current clinical and histopathological methods. These observations have a bearing on our surveillance of BSE-like disease in man in the coming decades and indicate the need for caution in interpretation until we have reliable markers for the causative agents of transmissible prion diseases.

Fears that BSE may be linked to CJD were increased in the mid-1990s by reports of CJD-like conditions affecting young people in the UK. With the exception of possible iatrogenic cases, sporadic CJD rarely affects people before the age of 30, but two cases of CJD and one CJD-like illness affecting three British teenagers reported in Britain in 1995[118] attracted the attention of the media (eg, *World in Action,* independent television program, UK, 14 August 1995). Three cases of CJD occurred in relatively young patients aged 19, 23, and 27 in Warsaw over a period of only a few years when the authorities were monitoring another form of brain disease. In recent years, there were separate reports of sporadic CJD affecting three British dairy farmers with one or more confirmed cases of BSE in their herds.[37,124,127] This was more than might be expected, although the occupational denominator used must affect the calculation. A fourth case of CJD disease in a beef farmer was reported in October 1995.[159] Some understandable alarm was expressed[4,61] and some moderation urged.[107] When associations were sought between CJD and the occupation of those affected, vicars and professional drivers also had a slightly higher experience of sporadic CJD than would be expected,[118,127] but the dangers of drawing conclusions from very small numbers were acknowledged. Significantly, when the agents from farmers who had had BSE cases in their herds were subsequently strain-typed, the strains affecting the farmers were not the same as the BSE agent.

CJD is not more common in abattoir workers, sheep farmers, veterinary surgeons, or butchers. If the BSE agent is the cause of sporadic CJD, these people could have been exposed occupationally to potentially infective tissues and would seem to be more at risk than dairy farmers. Evidence from the European Community surveillance project shows no differential increase in the risk of sporadic CJD to farmers in the UK resulting from possible occupational contact with BSE.[44] For example, between 1992 and 1994, there were three cases in British farmers, four in French farmers, two cases in Germany in a single year, and three in Italy in one year. If there is any link between BSE in cows and a CJD-like syndrome in dairy farmers, it might be reasonable to ask how the challenge that hit the cows might also have hit their close attendants. Would drinking unpasteurized milk merit consideration? This does not seem reasonable; there is no evidence of any infectivity whatsoever in transmission studies of milk from affected cows. The airborne route of infection or the contamination of wounds with dust from protein-supplemented cattle feed might merit investigation among those who milled MBM in the 1970s and early 1980s, if only to put another speculation to rest. It has been suggested that dairy farmers sometimes chew cattle-cake and might have ingested the agent in MBM concentrate. Pig and poultry farmers have not developed CJD although feed for these species would have contained more MBM concentrate than cattle feed when it was used. On present evidence, we have found no link between BSE and sporadic CJD. We must note that CJD seems to have a slight predilection for farmers, even in countries that have little or no BSE,[118] while those farmers who had BSE-infected cows and who contracted CJD in recent years in the UK did not yield an agent at postmortem with the characteristic profile of the BSE agent.

The theory that the whole problem of BSE in cows and BSE-like syndromes in their attendants can be attributed to organophosphate applications (used on sheep and cattle to control various infestations) is not generally regarded as tenable, but this view of Mark Purdey[116] was given considerable prominence in a September 1995 television program in the UK, and speculative suggestions that a toxic cofactor might be involved in BSE continue to be raised—but without persuasive evidence so far.

Despite the generally documented view that the agents of scrapie and BSE do not share common ground with the agent(s) of CJD, Diringer[48] considered that a link between scrapie and CJD is likely to exist. He reiterated the point that primary transmission of scrapie from sheep to cattle is likely to escape conventional histopathological diagnosis.[34] Diringer speculated that sporadic cases of CJD may arise in man indirectly as a result of passage of the scrapie agent from sheep through cattle to man, while a familial form of CJD (CJD_{200}) arises by a primary route *directly* from sheep in family members genetically predisposed to the disease because of an inherited mutation altering codon 200. He ascribed the rarity of expressed disease in man to the need for a two-hit situation in CJD_{200}—the genetic defect and the chance encounter with the agent—coupled with the 50% penetrance of the mutation, and the rare transmission of scrapie from sheep to another intermediate host (the cow, for example). He maintains that primary transmissions differ in clinical symptoms and histopathology from secondary transmissions and have longer incubation periods.

Strangely, we have not yet formally tested the general assumption that BSE was scrapie-derived by experimentally challenging cows in the UK with a range of scrapie strains. The technical problems were outlined by Collee and Bradley,[27] but there are pressing reasons to ask why such a study has been delayed for so long. The experimental primary transmission of scrapie to American cattle, reported by Clark and colleagues in the US,[23] resulted in a different form of BSE from that seen in the UK. According to Diringer, this is because the UK disease was caused by recycling, hence representing secondary transmission from BSE-infected material. He maintains that sporadic CJD and CJD_{200} are always due to transmission from animals to man and present with severe dementia as the predominant and early characteristic. On the other hand, he says, kuru and growth-hormone-derived CJD present with cerebellar signs of ataxia because "these two forms of disease are known to be caused by secondary transmission of the agent." He regards sporadic CJD as coming from sheep via cattle but, as it would then come as a primary transmission to man, he would accord BSE in man the clinical presentation of a primary infection. He uses the term *virus* for the transmissible agent involved.[49] It will be argued that, if Diringer's model has any validity, the outbreak of BSE in Britain has seen the possibility of a much more frequent oral transmission of the BSE agent to man in the 1980s so that sporadic cases of CJD will become more common.

In developing his theory here, the term *primary transmission* is used to denote that a new species is challenged, and *secondary transmission* to mean that the recently acquired agent is passed on within the same species. Thus man's first encounter with an animal-derived agent is primary and man-to-man passage of a recently animal-derived agent is secondary. (In the context of these slow infections, we shall have to define *recently* in the light of our continuing experience.)

Professor Diringer maintains that sporadic CJD and CJD_{200} are always due to primary transmission from animals to man and have severe dementia as the predominant and early characteristic. Until recently, the only known reservoir has been scrapie-infected sheep (and goats). He postulates that sporadic cases of CJD have resulted from passage of the scrapie agent from sheep to man—an extremely rare event in the general population. Now, with the advent of the UK outbreak of BSE, there is another candidate primary route—from sheep via cattle to man. The agent may have been modified by a period of secondary transmission within the bovine species during which Diringer suggests that the agent has "adopted a multipotent property of infection" as witnessed by its extended pathogenicity to exotic ungulates and members of the cat family and to some laboratory animals. He draws parallels with primary transmission across a species barrier with unadapted virus strains and secondary transmission within a species with adapted strains of viruses.

According to Professor Diringer, the scrapie agent in sheep hardly ever leads directly to disease in man, except in those with the codon 200 mutation, but the adapted agent in cattle is now capable of causing disease in other species, unconstrained by genetics. Diringer's theory implies that all humans, rather than only the genetically susceptible, could develop CJD when they encounter the bovine-adapted agent. In fact, the scrapie agent can be experimentally transmitted to many different species, more than the BSE agent at the time of writing. The BSE agent has transmitted naturally to a wider range of species than has the scrapie agent, but this is attributable to the common feed source of the BSE agent and thus delivery (in MBM or in infected carcass meat) to cattle, cats, large cats in zoos, and some exotic captive ungulates.

(*Note:* The possibility remains that BSE could have been transmitted back to sheep in infected MBM, but we have no present evidence.[27])

When CJD occurs as an iatrogenic infection following direct inoculation or implantation into the central nervous system (CNS), the syndrome is indistinguishable from sporadic CJD with progressive cerebral cortical dysfunction and dementia. Challenges delivered by a peripheral route, as in the repeated intramuscular injection of infected human cadaver-derived pituitary hormones used to treat growth defects,[18] resulted in progressive cerebellar ataxia akin to the clinical presentation of kuru. Accordingly, it was argued that if BSE caused a CJD-like syndrome in man as a result of ingestion or noncerebral parenteral implantation or injection, this would be a peripherally delivered challenge and a cerebellar syndrome, as in kuru (see below), might be the more probable presentation.[118,130] The cases of CJD-like disease in farmers and teenagers that attracted attention in 1995 in the UK did not present as cerebellar syndromes; this was thought to be reassuring.[130] However, according to Diringer's theory, the clinical presentation of BSE in man would not present as a cerebellar syndrome, and dementia would be the predominant clinical picture. In the event, the cases of new variant CJD that have been recognized to date typically presented with early psychiatric problems (see below).

57.5.3 The New Variant (nvCJD)

In March 1996, the CJD Surveillance Unit at Edinburgh reported that they had observed a (temporal) cluster of a new variant CJD syndrome (V-CJD, or nvCJD to denote new-variant disease) affecting 10 British patients aged less than 42 years (mean 27 years), with dates of onset of illness within the previous 2 years.[150] The nvCJD cases presented with early behavioral signs that were superseded after some months by progressive neurological dysfunction with ataxia and involuntary movements, and then there was progressive dementia with a clinical course ranging from more than 6 months to more than 22 months. There was thus a psychiatric presentation and a relatively prolonged CJD-like illness before death. The patients did not show the typical EEG findings of conventional CJD, but the histopathology of the brain showed spongiform changes and extensive abnormal PrP plaques. The psychiatric and neurological features now have been documented carefully.[161,162] The Spongiform Encephalopathy Advisory Committee (SEAC) stressed at that time that there was no evidence to link nvCJD with BSE, but in the absence of a credible alternative, the most likely explanation was that these cases were linked to exposure to BSE before the introduction of the ban on Specified Bovine Offals (SBO) of 1988–1989 that prohibited the use in products for human consumption of those tissues considered most likely to contain the infective agent. By the end of 1998, 38 definite or probable cases of nvCJD had occurred in Britain, plus 1 in France, and a link between nvCJD and BSE had been established by groups of workers using different approaches.[5,20,28,31,68] By the end of March 1999, a total of 39 deaths in the UK were attributed to nvCJD.

Lasmézas and colleagues in France successfully transmitted BSE to macaque monkeys in 1996.[92] The finding by Collinge and his coworkers in London in 1996[28,31] that different prion strains produce characteristic patterns of abnormal prion protein glycoforms on Western blots added to the growing body of evidence linking BSE with nvCJD. Preparations of prion protein from the brains of patients with nvCJD showed glycoform patterns similar to those seen in preparations from cows and other species with BSE and different from those seen with sporadic or iatrogenic CJD. These findings were developed further by the London team in parallel studies with transgenic mice expressing the human *PrP* gene and in nontransgenic mice. The results were published in October 1997.[68] The evidence that the agent of nvCJD is distinct from those of sporadic or iatrogenic CJD is compelling. Moreover, the glycoform profiles of the nvCJD and BSE agents are virtually identical, and there were striking similarities in the clinical presentations of the diseases that the two agents produced in mice and in the observed (histopathological) deposition of PrP in the brain. Minor differences between the two agents noted in transmission studies probably reflect a species barrier effect with the inbred mice used in these experiments; for example, nvCJD transmitted more readily than BSE to the human transgenic series.

The crucial clinching evidence of Moira Bruce and colleagues at Edinburgh, also published in October 1997,[20] "make implausible any assertion that all of these observations (of a link between BSE and nvCJD) are coincidental."[5] Transmission studies were done with material from six typical cases of sporadic CJD (spCJD) and three cases of nvCJD. The results were compared with the Edinburgh team's extensive experience of the "signatures" of a range of TSE strains in strain-typing experiments based on the incubation periods and pathology observed in panels of inbred mouse strains. Mice injected with material from the nvCJD cases developed a progressive clinical disease very similar to that produced by the BSE agent (at the lower end of the incubation period range for BSE and related isolates). Moreover, the lesion profiles for the nvCJD series—the patterns of vacuolar degeneration produced in the brains of the test mice—were those characteristic of the BSE agent. The team concluded that they now have "strong evidence that the same strain of agent is involved in nvCJD and BSE." Their strain-typing studies also showed that the same agent is responsible for FSE and for the TSEs seen in exotic ruminants in the UK. It is important that the agent of nvCJD can now be distinguished from that of spCJD and that the CJD illnesses studied in the two dairy farmers were of spCJD type and not nvCJD. Thus, while nvCJD is linked to BSE, the disease seen in the dairy farmers is not linked to BSE.

57.6 DISPUTED INTER-RELATIONSHIPS

Several important questions arise from the earlier kuru and CJD studies. Are the agents of CJD and kuru related to the agents of scrapie and/or BSE? These agents all cause what we have come to regard as prion diseases, but it is essential to be aware of the presently recognized domains of their inter-relationships. Thus, the agents of sporadic CJD and kuru may be closely related (if not identical), and the agents of scrapie and BSE seem to be closely related, although the BSE agent has a unique biological profile. A reassuring division seemed to separate the CJD/kuru complex from the scrapie/BSE complex; this is being further tested by comparing the biological characters in mice of agents isolated from recent and historical cases of CJD.[20]

The separation of CJD and scrapie is essentially based on epidemiological findings. There is much evidence (reviewed by Kimberlin[81] and simply summarized by Taylor[133]) to show that a country's experience of scrapie is unrelated to the numbers of cases of CJD that occur in its population. CJD has a worldwide distribution, but scrapie (and BSE) does not occur in Australia or New Zealand. It might be argued that CJD in Australia or New Zealand has its origins in the Old World, and that continuing immigration accounts for its continuing occurrence so that the

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absence of scrapie in Australasia may not be so significant in that part of the argument as it might seem. Moreover, Diringer's view is that we cannot rely on much of the epidemiological evidence of the past that seems to separate CJD from scrapie. He cites an 1883 report of scrapie in a cow[123] as evidence that this primary transmission from sheep to cow may have been happening for at least a century, and he considers that this is likely to escape conventional histopathological diagnosis.

Despite intensive surveys, there is no recorded occupational hazard for the development of CJD, for example in sheep farmers, butchers, veterinary surgeons, cooks, or abattoir workers. False correlations have been drawn with the eating of sheep's eyes by a group of Jews of Libyan descent living in Israel (who actually have a genetic predisposition to the inherited form of CJD). Foods with a doubtful association have included black puddings and veal and venison, but these seem to join the list of several possible red herrings that statistical manipulations tend to throw up one year and reject the next when the data depend on personal and family recollections of dietary preferences of relatively small numbers of cases.[118] The possible association with veal or venison merits continued surveillance, while bearing in mind caveats regarding the analysis of data based on dietary histories (for example, the apparent incrimination of liver consumption in the US that is not now regarded as significant).

Is BSE really scrapie in cows? The circumstantial evidence linking the agent of BSE to that of scrapie is persuasive. The infection that led to the outbreak of BSE is calculated to have had its beginning in the late 1970s or early 1980s, just when the rendering process for the production of ruminant-derived protein feed supplements was changed. It is reasonable to assume that high-challenge doses of the scrapie agent were thus allowed to be distributed to cows and calves in the UK. We know that oral challenge is an inefficient route of infection for the prion diseases. The significant changes in the rendering process included the omission of the organic solvent step and the reduction of one of the heating stages. The relatively short incubation periods of the BSE-like illnesses that affected some of the captive exotic ungulate animals subsequently fed with protein supplements in Britain suggest that these animals are unduly susceptible. Perhaps cows in the UK are particularly susceptible at a critical stage in their development as calves, but note that there are definitely adult-exposure cases in cattle, although calfhood exposure predominates (oldest case 18 years old).

When a breakthrough infection occurred in cows, there was recycling of rejected bovine carcasses and offals before BSE was recognized as a specific disease and the bovine-adapted strain would be selected and sequentially passaged. This would have increased the exposure of cattle to the agent and, theoretically, would be likely to increase its virulence (aggressiveness, neuroinvasiveness) for cows, although there is no evidence for this. The cycle would not be broken until the disease was recognized in 1986 and the facts established in the late 1980s. When appropriate steps were taken in 1988 (ruminant feed ban) and 1990 (SBO ban for animals) and a suitable time elapsed for these to take effect, the numbers of infections in younger animals began to diminish progressively by age class (in the mid-1990s). The bans were not as effective as they should have been at the beginning; they have been progressively enforced so that numbers of cases in cattle born after the 1988 ban (BABs) also continue to diminish.[70,111,130] These reassuring trends reflect parallel experience on a much more extended time scale with kuru, but bear in mind that the ancestry of the agents of kuru and CJD seems to be different from that of BSE and scrapie, as discussed.

It may be argued that BSE is not scrapie in cows but that it has always been a bovine disease unrecognized until the circumstances of the present outbreak brought it to light. The BSE agent has very much in common with the scrapie agents, but it has some characteristics of its own. It can be transmitted to sheep and causes a disease resembling scrapie. When it is transmitted to mice, it produces a typical syndrome, but attempts to transmit it to hamsters have been so far unsuccessful (Dr James Hope, personal communication). When a scrapie agent from sheep in the US was parenterally injected into cows, the resultant disease was "distinctly different from BSE,"[34,57,104] but Kimberlin has pointed out that the American strain of scrapie agent used was not representative of the scrapie strains likely to exist in many of the different breeds of sheep in the UK.[83] Moreover, Diringer[48] holds that the UK strain of BSE is a secondary bovine-adapted strain and thus differs from a (primary) scrapie strain. An important observation is that all of the BSE isolates from cows in Great Britain (and even Switzerland) produce a uniform pattern of disease when subjected to a standardized mouse bioassay. Whereas we can characterize many different strains of scrapie on the basis of their different incubation periods and slightly different pathogenesis and neuropathology in mice (under standard conditions), BSE isolates from cows have similar characteristics in mice regardless of the geographical location of the cattle or the year in which the agent was isolated. Thus, the BSE agent has a profile of its own that "breeds true" in cows and sets it apart from previously identified strains of the scrapie agent. Furthermore, the strain characteristics of TSE agents from domestic cats, a nyala, and a greater kudu and from a pig, sheep, and goat with experimental BSE are indistinguishable from the "UK BSE agent," despite these different species having different *PrP* gene sequences.[20] This does not break the postulated link between scrapie and BSE. If the BSE agent evolved in the cow from the ovine scrapie agent, the continuing selective pressure of passage in the new host species would ensure the emergence of a mutant (bovine-adapted) strain with a relatively short incubation period, and this would then dominate. Kimberlin has shown how this happens with a hamster model and the selection of a mutant strain of scrapie (263K).[83] Nevertheless, the possibility that BSE was not recently scrapie-derived is still a valid option.

If BSE existed in cows before the present outbreak made us aware of it, the clinically expressed disease presumably was uncommon. Wijeratne and Curnow[143] drew our attention in 1990 to the observations of Young and colleagues in 1983.[160] These workers reported that 4.3% of 10,458 animals culled from 24,800 cows in 80 Friesian dairy herds were removed because of their "undesirable temperament." Is this a further clue to what might have been happening in our herds before the 1980s when BSE was recognized? These animals did not show other signs of BSE, and many factors influence temperament in animals (and humans). Moreover, if a relatively avirulent endemic (enzootic) strain of BSE existed undetected in the past, it is presumably still with us in our herds across the world. Despite all of the increased awareness of BSE and worldwide surveillance of the last decade, there is no supporting evidence for this, but Marsh,[98] the SEAC,[130] and others[110] note that there may be a rare subclinical form of TSE in cattle in the US. See also Rampton and Stauber's more recent claims.[117] In the US, the Food and Drug Administration (FDA) was relatively late (mid-1997) in banning the use of slaughtered animal products (all animal protein except pure pork or pure horse) from cattle feedstuffs. The US livestock industry has had a voluntary ban in place since 1996; this official ruling is a delayed but wise precaution, although it does not appear to extend to pet food or to chicken or pig feedstuffs. Debated interrelationships here again merit concern. Cross-contamination in mills and feed stores from the latter sources may have been an important element in prolonging the UK experience of BSE. Collee and Bradley observed in 1997 that mammalian MBM was not completely withdrawn from UK farms until August 1996.[27] In view of the large amounts of MBM exported until 1990 from the UK and destined for pig and poultry feedstuffs abroad, and considering the large numbers of breeding cattle exported from 1985 to 1990 before controls operated, it is surprising that more cases of BSE have not been reported from mainland Europe and elsewhere abroad.

57.7 BASIC PRINCIPLES

How can we explain the story so far? Let us develop an argument from basic principles, while conceding that the agents of the prion diseases seem to break some of the generally accepted rules of microbiology. The production of an infection depends on the transmission of an adequate challenge dose of the infecting agent by an effective route into a susceptible host. Factors that then influence the outcome include the virulence or aggressiveness of the pathogen and the resistance or relative immunity of the host. Although some orally transmitted microbial infections can be established by a relatively small challenge dose of less than 100 organisms, as in bacillary dysentery or typhoid fever, several of the typical so-called food-poisoning infections (and intoxications) depend on a prior multiplication stage in food to deliver a very high challenge dose of bacteria or bacterial toxins. There are clear exceptions to this generalization; for example, extremely low numbers of some food-poisoning salmonellae caused a nationwide outbreak of disease principally affecting children in Germany in 1993.[94] In this context, note that very small challenge doses of salmonellae in chocolate or cheese (dietary items rich in lipid) are known to be effective on occasion. It is thought that this may be linked to protection of the infective agent by dehydration during processing and investment in a protective lipid envelope (Professor J.P. Duguid, personal communication). The quantity and the quality of the pathogen are thus important considerations in the development of a successful challenge, but susceptibility factors in the host are also of great importance, and the physico-chemical nature of the vehicle may be crucial. Bear in mind that the altered rendering process for the production of MBM for cattle allowed much more lipid to be present in the mix.

Our knowledge of quantitative aspects of orally delivered enteropathogenic or neuropathogenic viruses is inadequate and we have few models to guide us; however, our evolving knowledge of the prion challenge associated with kuru (in which secondary cases do not seem to have occurred) might allow us some insight in assessing the potential hazard of BSE as a possible foodborne infection for man. Again, Prusiner's caution to regard prion diseases as "pseudoinfections" and to beware of the limitations of conventional terminology and concepts is timely and must be taken seriously in this discussion. Moreover, we must again note that the CJD/kuru complex (with man as the natural host) still should be regarded as separate from the scrapie/BSE complex, unless Diringer's argument holds.

There is presently evidence that prions observe some rules of specificity and there are clear experimental examples of species specificity in the molecular biology of prion disease. Consider the de novo synthesis of prions in an experimentally challenged transgenic mouse, chimaeric for the donor and recipient host species—for example, a mouse with human and mouse *PrP* gene sequences. When this mouse is challenged with either mouse or human infectivity prions, the incoming prion selects the PrP of the donor type to convert to the abnormal PrP isoform; thus, the challenge prions seek out their own species PrP in this experimental model. Prions thus appear to carry information that predetermines the nature of the prions that they cause to replicate in a susceptible host. However, after serial passage in successive hosts of the same species, specific prions of scrapie seem to become adapted so that a standardized dose can produce a reproducible disease syndrome with a set incubation period in laboratory mice (though this may be influenced by the genotype of the host, which can moderate degrees of susceptibility).

Difficulties in transmitting experimental scrapie and BSE to various animal species indicate that species barriers operate.[130] The apparently high susceptibility of the captive exotic ungulates, especially the greater kudu, that went down with BSE in Britain, suggests that these animals are particularly vulnerable, or that they were challenged in a way that is unduly effective. As various species were affected, it seems likely that the nature (quantity and quality) and the mode of challenge were important factors. More species were similarly exposed but did not succumb. The kudu group seems to have been extremely susceptible, with possible lateral spread of the infection; this remained unproven in 1998, but it has led to speculation that there may have been an African link with the origin of BSE in the UK. Although spongiform encephalopathy has been recognized in wild and captive deer and elk in the US, I am unable to trace any record of BSE-like disease in native African animals.

The promptly effective control of the FSE outbreak in domestic cats in the early 1990s clearly suggests that the cat food manufacturers were quick to appreciate the problem and to exclude a suspected ingredient. We do not know if the absence of reported TSE in dogs reflects another particularly effective (canine) species barrier or whether the differences between commercially produced dog food and cat food are crucial in this respect.

The fact that, in general, only a few cows in any one herd have been affected by BSE is difficult to explain, although microbiologists and infectious disease physicians are well acquainted with the phenomenon in relation to diseases as different as meningococcal meningitis, pneumococcal pneumonia, and poliomyelitis in which carrier rates may be very high while apparently sporadic cases of the expressed disease occur during an outbreak. Explanations for such a hit-or-miss pattern of expressed infectious disease usually include considerations of variable genetic resistance, an unequal distribution of the challenging organism, changes to virulence that may occur within the host, failure of specific immune mechanisms in individual cases, and a compromised state that lowers the specific or nonspecific defenses of an unfortunate victim. In the transmission of BSE from contaminated feed to an occasional cow, having excluded genetic predisposition as a major factor, it seems likely that the challenge dose (in its unusually resistant form) was very unevenly distributed in the supplement given to a herd. It may be that the time of challenge of a calf is critical and that the agent could perhaps have a better chance of reaching its target if the permeability of the animal's gut is still in a relatively immature state. It might be that the weaning diet occasionally could damage the gut and allow a challenge access at a critical time or that a microbial infection or a chemical agent depressed natural defenses. It may be that the challenge dose was very small and was delivered over a prolonged period (say, from the late 1970s until the late 1980s, when the feed ban was introduced), so that one or other of these cofactors had to operate to produce the disease. And it may be that a small but potentially effective challenge might be associated with such a prolonged incubation period that the animal died or was killed before the disease was expressed. Considerations such as these allow innumerable speculations at this stage in our ignorance of the key mechanism of infection in BSE. Bear in mind that the evidence of many experimental models of prion disease indicates that the oral route is inefficient, but experience with the agents of kuru and other diseases in animals shows that a very high oral challenge dose can be effective.[58] (Although it is generally thought that kuru was transmitted orally in the Fore people, some writers reserve the possibility that the ritual smearing of remains of the deceased on the skin and around the eyes of the mourners, particularly the children, may have been the significant route of infection.) The most likely explanation for the low within-herd incidence of BSE is that there was low average exposure of the cows to "packets" of infectivity that were widely spaced early in the epidemic, reached a peak until controls became effective, and are now declining to zero.[85]

Is it possible that when BSE occurs in one animal in a herd, all of the cows have actually acquired the agent in a low-grade, unexpressed form that may smolder in the whole herd? The concept of a temporary carrier state is valid for diseases such as poliomyelitis and pneumococcal pneumonia. The carriage of the organism at some location in the host quickly wanes if the disease is not expressed. In some diseases, such as typhoid, a chronic carrier state may develop in a patient who has survived the expressed disease. But a more insidious form of prolonged subclinical infection, termed latency, is seen with herpes virus infections and some other viral diseases. Here, the infecting agent becomes integrated into host cells and has the capacity to replicate and emerge from latency to produce disease when circumstances alter the host's ability to hold it in check. Professor Lacey[91] has developed an argument along these lines for BSE, but we have no evidence for this. With regard to lateral spread, we do not seem to have any evidence for excretion of the BSE agent from noncalving infected cows, whether affected or not. The situation in sheep scrapie is different.

Kimberlin and others entertain the possibility that a very low-dose challenge with BSE in cows may have a very extended incubation period, so that the real mortality is not likely to be revealed because most cows are not allowed to live long enough for the full picture to emerge. Lacey has joined up a series of the above speculations to produce his doomsday scenario; some of the links in his logic are vulnerable, but they may be redeemed by Diringer.[48] The estimates developed by Dr Dealler[39,41] assume that perhaps 700,000 undiagnosed BSE-infected cattle went into our food chain before our control measures were effective. We urgently need a more sensitive assay system to investigate each of the theories further, and we must meanwhile preserve a rational objectivity. If the change in rendering practices with noninactivation of prions in MBM does not seem to be the entire reason for the start of the outbreak in cattle, it may be that the effective oral dose in ungulates needs to be at a critical level and that partial inactivation in the past has been enough to avoid clinical disease. When a combination of recycling of infected carcasses and modified rendering brought together very high-challenge doses and significantly lower inactivation, the critical level of challenge may have occurred in the packets of infectivity that we envisage in MBM.

A worrying feature of the species barrier is that a prion disease affecting species A that cannot be transmitted to species B may be established successfully in species C and then, after passage to species C, may possibly be transmissible to species B. Thus, it is important in the control of the potential hazard of BSE for man that we do everything possible to suppress the disease in cattle and in any other species. The ban on ruminant-derived protein substances in food destined for cattle in 1988, the compulsory slaughter and complete destruction of BSE suspect cattle, and the ban on the use of SBO in human food in 1989 and its extension in September 1990 to apply to food for all animals and birds in the UK were major steps toward safety in this context. The lesson should be further explained to any countries that do not yet see the writing on the wall, and the message to scientists working with BSE in experimental animals must be very clear.[1] European countries are well informed of the British experience via the European Commission. Other countries are informed on animal health matters by the Office International des Epizooties and on public health by the World Health Organization.[101,102] Although BSE has not been reported in the US, the authorities there are well informed,[8,110] but the source of TME merits further investigation there,[98,130] the feeding of ruminant-derived protein to cattle should certainly be banned (this happened in the latter part of 1997), and increased surveillance of "downer" cows will be a wise precaution. Meanwhile, for very important economic reasons, Argentina, Australia, New Zealand, and many other interested countries are maintaining their vigilance.

Prusiner considers that the species barrier is determined by the degree of difference between the amino acid sequence of the PrP of the donor species (the challenging prion) and that of the recipient species.[115] The closer the resemblance, the more likely it is that the prion disease will be transmissible. Bovine and ovine PrP differ at 7 positions, whereas bovine and human PrP differ at more than 30 positions. He argues that the likelihood of transmission from cows to man must accordingly be low, but he adds a cautious afterthought. It may be that some areas of the PrP molecule are more important than others in terms of their sensitivity or resistance to the induced conformational change that produces the abnormal form. Thus, there may be critical hot spots, perhaps near the center of the PrP molecule, where similarity of amino acid sequence reduces the efficacy of the species barrier. These considerations have become even more complicated with the finding that helper molecules made by the host may hinder or assist refolding of the PrP molecule. Almond[4] looks less optimistically at this aspect of cross-species transmission. He cites the work of Scott and coworkers[125] and holds that the bovine *PrP* gene sequence is slightly closer to the human sequence than is the sheep sequence, and the apparently important 96 to 167 region has only five differences as compared with six.

57.8 WHAT HAS BEEN DONE TO BRING THE UK CATTLE OUTBREAK OF BSE UNDER CONTROL?

The short answer is a great deal, but some of the legislation was not enforced quickly enough. Recognition of the disease in 1986, followed by good communications between the veterinary authorities and cattle breeders and an immense amount of investigative work in Britain, led to general awareness of the situation and some inspired retrospective analyses. BSE was made notifiable in June 1988. An initially inadequate compensation scheme was introduced, and the terms were subsequently improved. Compulsory and controlled slaughter of all animals with suspected BSE was arranged, and the laboratory confirmation of all suspect cases was organized. A ban on the use of milk from affected animals was introduced and instructions for the safe disposal of carcasses were issued. Orders were issued to control possible infective hazards during the calving of parturient suspect cows isolated on approved premises and with disposal of placentas and bedding by incineration and burial and with disinfection before reuse of the premises. More effective preventive measures were needed. On August 15, 1995, a new SBO order imposed further restrictions with the compulsory staining and disposal of specified bovine offal. Significantly, skulls may not be opened to remove brains and eyes; there are now restrictions on the mechanical recovery of meat from vertebrae,[27] and bovine meat on the bone (and bone marrow) are banned as from December 16, 1997.

A remarkably comprehensive investigation into possible sources of the outbreak of BSE was set up in the early days. In the first instance, this explored theoretical causes across an enormously wide range from chemical poisons and trace element deficiencies to genetic diseases and vaccine reactions. The evidence incriminating ruminant-derived protein in animal feed supplements was sifted and collated painstakingly, with many cautions and provisos underlining the scientific care of those involved in interpreting the clues. All of this was done efficiently, despite unappreciative and sometimes ill-informed and alarmist comments in the press. Official action followed a rational course that has been held firmly [(see the Spongiform Encephalopathy Advisory Committee's Interim Report on Research (1992)[129] and the Progress Reports of the Ministry of Agriculture, Fisheries and Food (1995)].[111] A summary of the early steps is given in Table 57–3.

Investigations have continued and special efforts have been made to evaluate the gradual success of the ban on the use of ruminant-derived protein supplements in animal feed and to extend the search for other risk factors that might suggest other supplementary routes of infection.[71,129,130] Some unconfirmed cases of clinically suspect BSE in cattle may be attributable to other diseases; these are under surveillance.[140] The media have been vigilant in asking pointed questions and getting authoritative answers;[73] there has been a considerable amount of irresponsible reporting, and there has been some evident reluctance to put all of the facts on the table. However, it is difficult to understand or to accept all of the charges of official silence and inadequacy,[91] although the management and control measures could have been better at some critical stages.

57.9 WILL THINGS NOW GET WORSE FOR THE COWS?

In 1991, I set out the likely course simply as follows[25] on the basis of data and predictions published by several experts. If the pattern of disease originally recorded by Wilesmith and the Southwood Working Party is followed, most cases of BSE will occur in cows that were infected as calves in 1987 and 1988

Table 57–3 Early Official UK Action Taken To Monitor and Control BSE

Date	*Action*
November 1986:	BSE recognized and histologically confirmed in a British cow.
November 1987:	Occurrence of new disease of BSE reported by Wells and colleagues in the *Veterinary Record.*
May 1988:	Working Party on BSE set up under Professor Sir Richard Southwood to examine, report, and advise on implications for animal and possible human health hazards.
June 1988:	BSE made notifiable in Britain.
July 1988:	Ban on sale or supply of ruminant-derived protein for feeding to ruminants (Ruminant feed ban).
August 1988:	All clinically suspect animals to be slaughtered; carcasses to be destroyed; 50% compensation paid.
December 1988:	Milk from suspect animals to be destroyed.
February 1989:	All recommendations of Southwood Report acted upon.
June 1989:	Consultative Committee reports on research needs.
November 1989*:	Ban on sale for human consumption of specified bovine offals in which the BSE agent might occur (Specified Offals ban). (Note: Offals from calves aged less than 6 months were initially exempted from this ban. In June 1994, the ban was extended to include intestine and thymus from calves aged less than 6 months.)
February 1990:	Compensation for slaughter of suspect animals increased from 50% to 100%.
April 1990:	New Spongiform Encephalopathy Advisory Committee (SEAC) set up under Dr David Tyrrell to advise the British Ministry of Agriculture, Fisheries and Food, and the Department of Health.
September 1990:	Specified bovine offals ban extended to embrace all animal feed and poultry and bird feed as well as human food.
October 1990:	Cattle farmers to keep records of all calves born, and the identities of the dams.
July 1991:	Export controls imposed on specified bovine offals (SBO).
November 1991:	Ban on use of SBO for production of fertilizers.
August 1995:	SBO Order imposed further controls on specified bovine offals.

*Dates for equivalent orders in Scotland and Northern Ireland are slightly different.

and will develop signs of illness 3 to 5 years later, say 1990 to 1993. The numbers of cases of BSE greatly exceeded those originally forecast by the Southwood Working Party,[119] presumably because of the recycling that occurred before the feed ban was introduced; this could not be taken into account by the Southwood predictions because the amount of recycling could not be estimated. The numbers of confirmed cases peaked in 1992 to 1993 as Wilesmith predicted, with substantial decreases through 1994 and into 1995. On the basis of continuing advice and surveillance, I then wrote in 1993[26] that cases of BSE infected in 1987 to 1988 (before the ban) will be clinically evident in the years 1989 through 1995 or beyond, but most will have occurred by 1994 and 1995; there would be many cases after the average 4- to 5-year incubation period had passed and allowances would have to be made for cases that could occur from contaminated feed that was already in the pipeline when the ban was imposed in 1988. This, and other intentional or inadvertent slippage, accounts for many cases that could arise in animals younger than 4 years old in 1993 (ie, animals born after the 1988 ban on the sale or supply of ruminant-derived protein feed supplements).

With hindsight, it would have been wise to combine the ban on the sale, supply, or use of these supplements with rigorous steps to ensure that cross-contamination of other feedstuffs could not occur and that contaminated feedstuff in the pipeline or in store on site was identified and destroyed. Subsequent legislation has gone some considerable way to close various loopholes in this regard. Substantial numbers of BABs developed BSE (Table 57–4); up to June 1, 1997, 32,767 were confirmed. By the end of 1998, the number had risen to 37,987. Most of these had presumably ingested ruminant feed protein supplement. The numbers affected are considerable and the annual occurrence of these cases would be expected to reflect a mean incubation period of about 4 to 5 years, with a decreasing chance of infection for each annual cohort as the controls became more effective. Thus, we might expect a major decrease in the numbers of BAB cases of BSE in calves born, say, after 1994. At this stage, it is too early to cheer. The evidence that there is occasional vertical (ie, maternal) transmission of BSE from a cow to its calf complicates the picture, and the possibility that lateral (ie, horizontal) infection may rarely occur (from one animal to another by contact or by environmental contamination) is still entertained though unproven. Although it is possible that BSE is occasionally transmitted maternally or horizontally,[7,130] the indications are fairly clear that these events are not occurring sufficiently often to sustain the present outbreak. If feed is the only significant source of infection (and no other source has so far been identified in a very extensive research program), and if current controls really are applied, it is expected that the numbers of new confirmed cases of BSE will continue to decline. The data available in mid-1998, when this review was completed, support this prediction, but it has to be admitted that some cows with early signs of BSE were being missed before the controls were reviewed and tightened in the mid-1990s. With this in mind, for example, Hoinville and colleagues in 1995 took account of unidentifiable infected animals that had been culled before they exhibited clinical signs of BSE.[71] Stekel and colleagues[132] have predicted future numbers of cases of BSE in cattle (Table 57–5).

Table 57–4 Confirmed Cases of BSE in Cattle Born after the July 18, 1988 Feed Ban, by Date of Birth

Year of Birth	*Number of Cases*
1988 (after July 18)	11,917
1989	12,578
1990	5,535
1991	4,279
1992	2,417
1993	1,121
1994	142
1995	3

(From data available up to January 1, 1999 [37,987 cases])

Source: Data from *MAFF Progress Report*, December 1998.

The crucial factor in reducing the numbers of further cases (BABs), which already may be quite numerous, is the exclusion from the animal food chain of tissues known to contain the BSE agent in infected cattle. Collee and Bradley[27] summarized anxieties relating to the industry's carelessness and evasion and delays in appreciating some of the hazards of cross-contamination of animal feedstuffs; as late as mid-1994, it was estimated that about half of the specified bovine offal materials being processed were not separated properly and could still have been entering rendering plants. The Meat Hygiene Service was set up in early 1995 to make the UK controls more effective. There was much to do. Table 57–6 shows the new measures that were introduced from late 1995 to 1998.

Confirmed cases of BSE in cows born after the mid-July ban in 1988 were worryingly numerous. The data given in Table 57–4 must be interpreted with caution because the deaths of all of the cattle born in a particular year and destined to die of BSE

Table 57–5 Predicted Numbers of Cases of BSE in Cattle in Great Britain

Year	*Predicted Numbers of BSE Cases (Low and High Estimates)*
1995	13,880–16,110
1996	8,050–11,270
1997	4,250–7,130
1998	1,970–3,870
1999	840–1,880

Source: Data from D.J. Stekel, M.A. Nowak, and T.R.E. Southwood, Prediction of Future BSE Spread, *Nature*, Vol. 381, p. 119, © 1996.

Table 57-6 Later UK and EC Action To Prevent BSE from Entering the Human Food Chain: A Simplified Account of Relevant Regulations (1995–1998)

Date	*Action*
15 December 1995	UK SBO Amendment Order essentially prohibited use of bovine vertebral column for products destined for human consumption, including mechanically recovered meat (MRM).
27 March 1996	EC ban on export from UK of live cattle, semen, or embryos; ban on bovine meat or products from cattle slaughtered in UK that could enter animal feed or human food chains; ban on bovine materials destined for use in medicinal products, cosmetics, or pharmaceutical products; and ban on mammalian-derived meat-and-bone meal (MBM).
29 March 1996	UK BSE Amendment Order prohibits sale or supply of any mammalian MBM or any feed containing it for feeding farm animals, horses, or farmed fish.
29 March 1996	Specified Bovine Material Order replaced UK SBO Order of 1995. Whole head of all cattle aged >6 months to be regarded as SBO (except tongue).
3 April 1996	UK 30-month Slaughter Scheme: All bovine animals >30 months at time of slaughter must not enter human food or animal feed chains.
4 April 1996	UK cattle identification regulations extended.
1 May 1996	Specified Bovine Materials (SBM) to be removed from carcasses and handled separately. UK Regulations on rendering and disposal brought into line with EU requirements.
1 May 1996	UK Regulations made more rigorous and applied to approved operators of incinerators or rendering plants and to licensed operators of licensed abattoirs.
10 June 1996	UK Feed Recall Scheme to collect and dispose of MBM or feed containing MBM still on site at farms, mills, or merchants' premises.
11 June 1996	EC ban on gelatin, tallow, and semen partially lifted.
1 July 1996	UK Cattle Passports Order requires mandatory cattle movement documents in GB for all cattle born from this date. (Computerized tracing planned to be on line by 1998.)
1 August 1996	UK Prohibition on possession of MBM where any livestock feed is kept; requirement for disposal and recall of MBM and cleansing and disinfection of places, vehicles, and equipment that might have been contaminated with MBM.
15 September 1996	UK ban on sale for human consumption of any part of the head of a sheep or goat (except the tongue).
16 December 1996	UK announces selective cull of cattle deemed most at risk of BSE.
24 December 1996	UK requires all UK plants producing gelatin from non-UK material to be registered.
16 December 1997	(UK) Deboning of all beef from all cattle aged >6 months at slaughter now required before sale to consumers.
22 December 1997	BSE Inquiry announced in UK.
1 January 1998	UK controls adopted on Specified Risk Material in line with EC recommendations.
15 April 1998	UK regulation: Cattle born after 1/1/98 must have a MAFF-approved eartag in each ear and a cattle passport.
29 July 1998	UK: Compulsory slaughter of scrapie-affected sheep and goats.

Source: Data from *MAFF Progress Report*, June 1998 and MAFF *News Release* 288/98 of July 8, 1998.

cannot be recorded until the full range of the incubation periods has run. Even then, we have to take account of possibly significant numbers of unrecognized but infected animals that are slaughtered or die for other reasons in the meanwhile. Further work is needed to determine, now, whether lateral transmission might account for some of the late cases and whether other factors are operating. If a further BSE mutant arose with an increased capacity for maternal or lateral spread among cattle, this might dramatically alter these forecasts, and additional controls would be necessary. Nevertheless, continuous study of the pathology of BSE and of the BSE agent's biological characteristics so far reveals that a single strain is responsible.

57.10 BSE IN MAN

57.10.1 What Is the Risk of Foodborne Transmission of BSE to Man?

It is not possible to give a direct answer, but it is helpful to consider the protective factors that may operate. All of the points reviewed above allow us to compile a list of reasons why man may be protected from the full effects of the BSE hazard. This analysis started with the opinions of the Southwood Committee[119] and was followed by others, including Taylor's careful assessments[133,134] and the views of Harrison and Roberts[65] and Will.[148] Many of the points were summarized in reviews of the evidence by Collee,[24–26] by Bradley and Wilesmith,[13] and Collee and Bradley.[27]

First, there was the concept of the species barrier with "interspecies variations in the prion gene so that differences between the encoded proteins may make prions (in the challenge) less able to modify the normal PrP of the host."[65] There are several variable components in the species barrier: variation in the *PrP* gene sequence of the donor and the recipient species, and the strain of the infecting agent.

Second, the oral route of challenge generally is regarded as very inefficient in this context; we have to look as far as the models of kuru and TME and BSE-like disease in one of the species of captive exotic ungulates to find evidence of apparently regular transmissibility by the oral route, and there are some doubts connected with each of these examples. In studies with orally delivered challenges fed to experimental animals, successful transmission was generally associated with very large doses.[9,58] Some captive wild Felidae that succumbed to nonexperimental oral infection may have consumed high-titer CNS tissues from cattle infected with the BSE agent. Other species were presumably similarly exposed and, so far, they seem to have been resistant. On the other hand, some captive bovids fed MBM supplement were highly susceptible. The outbreak of FSE possibly caused by transmission of the agent of BSE in pet food to domestic cats in Britain has provided an instructive model.

The third point is that many mouse bioassay experiments to determine the infectivity of tissues from cows with BSE indicate strongly that the BSE agent is not so widely distributed in detectable amounts in the tissues of the cow as the scrapie agent is distributed in sheep tissues.[82,83] High infectivity in sheep scrapie is associated with the brain and spinal cord, with medium infectivity in the spleen, tonsil, lymph nodes, distal ileum, and proximal colon. Infectivity also has been reported in the retina.[111] No detectable infectivity was recorded for skeletal muscle, milk, blood, and various other tissues. Similar studies with tissues from BSE-infected cows have, so far, indicated that infectivity is associated only with the brain and spinal cord; the assays were performed in mice by parenteral inoculation. The infectivity of the brain and the noninfectivity of some of the other tissues also was demonstrated by oral challenge experiments.[103,111,130] A very important point is that mice exposed to parenteral challenges with muscle, spleen, and placenta from BSE-infected cows did not show any evidence of a spongiform disease within their natural lifespan.

Until now, estimates of tissue infectivity in various experimental animal models have been based mainly on tests of infectivity in mice (mouse assay). With the subsequent realization that the mouse assay (ie, using intracerebral inoculations in a heterologous species to test bovine tissues) is perhaps 100–1000-fold less sensitive than an equivalent assay in the homologous species (ie, intracerebral tests of BSE-infected bovine preparations in calves), there has been a reconsideration of our views on the relative infectivity of various tissues in BSE. The mouse tests had indicated that the BSE agent is much less widely distributed in the tissues of infected cows than the scrapie agent in sheep. In naturally infected cattle, BSE infectivity as judged by mouse assay seemed to be restricted to the brain and other central nervous tissue and retina. Tests with high oral challenges in experimentally infected calves have since shown possible infectivity in distal ileum,[139] and, more recently, in bone marrow and dorsal root ganglia.

The widening concept of the potential infectivity of BSE-infected tissues from cases of the disease in

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animals and man has driven the authorities to introduce additional safety measures. These have included a ban on the sale for human consumption of beef on the bone and the exclusion of bovine bone marrow and dorsal root ganglia.

Of course, legislation and guidelines adopted in Britain and guidelines adopted in other countries already provided some possible protective cover.[111] For example, the various measures outlined elsewhere in this chapter to recognize and eliminate cases of BSE from the food chain and the imposition of the specified offals ban (with its further extension to apply also to calves less than 6 months old in regard to the intestine and thymus gland) should have further reduced the chances of the delivery of a significantly high oral challenge with the agent of BSE in our diet.

It is likely that man has been challenged orally with the scrapie agent in some sheep products for more than 2 centuries, but there is no recognized association between scrapie and human disease over that long period. The early evidence seemed to indicate that the BSE agent is a form of the scrapie agent that achieved a breakthrough in the cow. If we accepted this view, we would have to assume that a species barrier between the sheep and the cow, which had held for centuries, was broken by the "unnatural" circumstances that allowed overwhelming challenges to be delivered to herds of British cows in the period from the late 1970s until at least 1988. As we have discussed, the breaking of a species barrier and the adaptation of a pathogen to the new host has the potentially dangerous implication that other species barriers may be less secure; accordingly, we must proceed with caution—especially in the light of Diringer's theory.[48] Moreover, the back-transmission of the BSE agent from cows to sheep merits consideration,[27] and this is now under investigation, with some precautions already in place. Scrapie in sheep in the UK was only made notifiable in 1993, and compulsory slaughter of sheep (and goats) with scrapie was not introduced until 1998. Items presently banned for human consumption include the heads and spleens of all sheep. In the case of sheep more than 1 year old, vertebral columns are banned and meat recovered from vertebral columns is banned.

If BSE existed in cows (enzootically) before the present large-scale outbreak, there is little doubt that the factor precipitating the UK catastrophe was again the alteration of the rendering processes, and that the vehicles of the severe challenges were ruminant-derived protein supplements produced by these processes. It was thus necessary, in the late 1980s when BSE was recognized, to bring in severe measures to protect the public health in the UK against the theoretical hazard of ingesting high challenge doses of the BSE agent in bovine products.

Harrison and Roberts in 1992 regarded the BSE outbreak as "an accidental experiment on the dietary transmissibility of prion disease between sheep and cows."[65] These authors considered that a subsequent experiment on the transmission of BSE from cow to man probably occurred from 1986 to 1989, because BSE-contaminated products may have entered the human food chain at that time. They regarded the chance of BSE causing human disease as extremely small. We can add, again with hindsight, that the early recycling of affected cows in the process must have fueled the outbreak that either began with an ovine strain that became bovine-adapted, or else began with the boosting of a previously undetected (enzootic) bovine strain that was already bovine-adapted. Hence, the early 1980s saw the beginning of an extended "natural" experiment in cow-to-cow transmission of BSE (via rendering), and the mid-1980s saw the start of other natural experiments in direct transmission by possible vertical and lateral spread of BSE within affected herds.

Our experience in the transmission of prion diseases indicates that the oral route of challenge is much less effective than parenteral routes. Fortunately, the majority of the bovine-derived materials used for (parenteral) implantation or injection were produced from tissues or fluids that have been shown to contain no detectable BSE infectivity by bioassay in mice, but some preparations derived from bovine neural tissues have been used parenterally in the past[10] and some have been implanted intracerebrally in neurosurgical procedures. If, in future years, evidence links these with a BSE-like disease in man, it will be important to bear in mind that the risk associated with oral challenges is still likely to be much less by several orders of magnitude.[83,148] It will also be important to remember that our presently heightened awareness of the prion diseases will ensure that human encephalopathies and dementias will be reported and investigated more extensively in the next decade.[17,53,63,78] Until we have more specific markers for these diseases, we must guard against automatically assuming that, for want of a definitive diagnosis, the agent of BSE in Britain (and elsewhere in the world) is responsible for all human spongiform encephalopathies. Will, in 1991, considered it essential to minimize human exposure to the BSE agent in food and summarized the evidence for his view, at that time, that the consumption of beef (bovine muscle) is exceedingly unlikely to be hazardous.[148] The risk of foodborne transmission of BSE in the US was regarded by Moon as even lower than the low risk in the UK.[104] Cooking temperatures do not definitely inactivate the agents of scrapie or BSE, but they can reduce the titers of infectivity.[133,135] In the UK, and in any country in which BSE has been diagnosed in indigenous cows, it is reasonable to avoid eating bovine tissues in which high concentrations of the BSE agent are known to occur when a cow goes down with BSE (notably, brain and other specified offals that have been banned in the UK). There are justifiable worries about instances of carelessness, negligence, and fraud in British slaughterhouses and farms (*The [London] Times*, December 2, 1995) that may have frustrated our protective legislation in this context. When this chapter was completed in September 1998, the UK government had tightened regulations with further control measures (Table 57–6). After December 15, 1995, products destined for human consumption derived from bovine vertebral column, including mechanically recovered meat from this site, were banned. For operators outside abattoirs such as those in knackers' yards and hunt kennels, the whole backbone (apart from the tail) of a cow was to be removed and regarded as prohibited offal. This was because some abattoirs were leaving parts of the spinal cord attached to the carcass. Subsequently, the whole head (excluding the tongue) of cattle aged more than 6 months old was to be regarded as SBO. (The term SBO was replaced and extended by the term *specified bovine material* [SBM], on March 29, 1996.) Concern continues to be expressed about beefburgers and other processed meat products (pies, beef soups, stock cubes, and patés) in which the use of cattle brain and specified offal has been banned since 1989 to 1990, although brain from calves under 6 months of age still can be used. It may seem unreasonable that calf brains are still allowed into the food chain (*The [London] Observer,* Sunday, December 3, 1995). In a country with a continuing outbreak of BSE, some of us feel that it would be wise to ban the supply of bovine brains, spinal cord, and offal from cattle and calves of any age for human (or animal) consumption. The facts of the matter are, however, that data from a careful series of pathogenicity experiments with orally challenged calves showed no detectable infectivity in brains from these animals at least up to 22 months of age and 18 months after challenge. On April 3, 1996, the UK 30-month slaughter scheme ensured that all cattle over 30 months old at slaughter must not enter the human food or animal feed chains. As time goes on, cohorts of cattle aged up to 30 months are progressively more unlikely to have been exposed to infected feed, especially with the introduction of measures to recover MBM from sites indicated in Table 57–6 and to clean and disinfect these sites and equipment. The numbers of calves that may continue to be infected by maternal transmission also will decrease, and this may be accelerated by the selective cull introduced in December 1996. With the UK banning of bovine meat on the bone and bone marrow in December 1997, attention is again focused on products that are heavily dependent on bovine bones as source materials.

Gelatin. Gelatin extracted from bovine skin and bones is used widely in catering and confectionery and in the production of some pharmaceutical preparations, but is considered unlikely to be contaminated with the BSE agent,[101] although the US authorities presently are unconvinced. The Scientific Veterinary Committee (ScVC) in Brussels and others recommend that skulls and vertebrae should not be used as source materials for gelatin because there is a risk that infected brain or spinal cord could be present. Concentration of such infection could be important, even if the processing is rigorous. If tallow and gelatin destined for incorporation in human foods or medicines are derived from BSE-free cattle from closed BSE-free herds that had not been fed ruminant-derived protein, this would make assurance doubly sure. In February 1998, the EU Scientific Steering Committee (SSC) reported their discussions on gelatin and reached the following scientific opinions.

1. The preparation of gelatin and acceptable sources for human consumption or for cosmetics were defined. Two critical considerations are the traceability and treatment at the source of the raw materials, including the meticulous removal of specified risk materials. The SSC acknowledged the FDA (1997) opinion that gelatin can be produced safely from bovine hides from any country, provided that the hides have not been contaminated with specified risk materials and that hides from cattle with signs of neurological disease are excluded. The SSC considered that gelatin for human consumption could be prepared from bovine bones and skins without removal of specified risk materials if obtained from countries known to be BSE-free or "at negligible risk." In the case of sourcing from "lower risk countries," specified risk ma-

terials should first be removed and the origin should be certified to be exclusively from animals deemed fit for human consumption. For raw materials from "high-risk countries," and with exceptions restricted to special cases, only hides should be allowed and, even then, they must be from animals fit for human consumption. Materials sourced from countries of unknown status might be judged on their specific merits or might be considered as for those from high-risk countries.

2. Gelatin destined for pharmaceutical products and parenteral use was considered separately, and subclassified as (a) gelatin for oral or topical (excluding ophthalmic) use, and (b) gelatin for parenteral or ophthalmic administration or for use in implantable devices. For oral or topical use, the conditions set for food and cosmetic use should be met, but with special grade gelatin considered for topical products that might be applied to large areas of damaged skin or open wounds. Gelatin for parenteral, ophthalmic, or implantable use should be of special grade with appropriate purification procedures followed in its production. If gelatin is to be used as a reagent in the manufacture or production of pharmaceutical preparations, the more stringent controls should apply. Gelatin destined exclusively for industrial use in the production, for example, of photographic products and for various technical applications, should be prepared by an appropriate production process within the SSC guidelines; if ingestion of the gelatin or human exposure to it may be expected under normal conditions of use, the relevant conditions should apply. The original papers should be consulted for details of these careful and complex deliberations (see Institute of Food Science & Technology [IFST] [UK] at http://www.easynet.co.uk//ifst).

The emerging new evidence of BSE infectivity in the bone marrow and dorsal root ganglia of experimentally infected calves had repercussions, but the guidelines are presently held. Note that the recognized manufacturing process for gelatin includes at least four steps that each are known to reduce the titer of infectivity of spongiform encephalopathy agents. The initial degreasing step has an estimated reduction factor of about 100. Acid demineralization also has a factor of 100. Alkaline purification is even more powerfully inactivating with a factor of 1,000; and the sterilization process has a mean value of 100. As a result, the titer of infectivity of the raw material is said to be "probably reduced by a factor of the order of a thousand million (10^9)." There are areas of uncertainty in these calculations; for example, definitively sterilizing conditions for TSE agents in a proteinaceous mix would be likely to denature gelatin or at least alter some of its properties. Significantly, the UK gelatin manufacturers have decided to manufacture gelatin for edible or pharmaceutical use from raw materials derived entirely from non-UK sources.

Tallow. The SSC performed a similar risk assessment for tallow. An appropriate purification process was outlined with special reference to time, temperature, and pressure parameters and with conditions known to be effective in inactivating TSE agents achieved throughout the material being processed. Emphasis again was placed on the importance of the traceability of the raw material and its treatment at the origin, including the removal of specified risk materials. In the case of tallow destined for human or animal consumption or application, the source animals should be fit for human consumption. If the material is from countries with no BSE or classified as at negligible risk, processing can proceed permissively but with the precaution that the purification process should be appropriate and validated. If sourced from "lower risk" countries, the use of specified risk materials should be excluded and the processing should be validated. If the country of origin is deemed to be in the high-risk category, specified risk materials should be excluded, the purification process should be validated, and the product should be subjected to 133° C for 20 minutes at 3 bar pressure or an equivalent TSE-inactivating procedure. Source countries of unknown status should be assessed on their individual merits or regarded as high risk. A ban imposed by the European Commission on the export of tallow produced from cattle slaughtered in the UK was partially lifted in 1996. Regulations implementing the conditions came into force in the UK on March 15, 1997, and a further UK SBM order was made on March 28, 1997. This legislation required that all UK tallow for food, feed, pharmaceutical, medical, and cosmetic uses must be manufactured to export standards in registered plants and must be derived from raw materials free from SBM and specifically free from bovine vertebral column. The regulations also require manufacturers using UK-produced tallow in the production of food, animal feed, cosmetic, medical, or pharmaceutical products to use only tallow produced in a registered plant. European Community inspections of UK plants are to proceed now, with a view to allowing exports of UK tallow to be resumed.

Beef. With special reference to the consumption of British beef, there is now much evidence that the BSE agent is not present in detectable amount in the muscle (meat) of an infected cow, but dorsal root ganglia and bone marrow must be excluded. These recent findings accord with Aguzzi's concept of PrP expression in peripheral tissue as a requirement for pathogenesis[2,11] and oblige us to look again at the nerve content of the muscle of an infected animal. Note, in this context, that the limit of sensitivity of the intracerebral mouse bioassay on which our BSE detection methods presently depend is uncertain, but it is much more sensitive than any other practical method currently available and is perhaps 200,000 times more sensitive than the oral route.[83] The cow presumably would be the most sensitive bioassay species for BSE (and perhaps transgenic mice with bovine *PrP* genes). Inevitably, a cattle-based assay would take years, and the development of a practical transgenic mouse assay requires much work. Advanced bioassay work currently in cattle and mice at the Central Veterinary Laboratory, UK, (Ray Bradley, personal communication) confirms that tests in cows are much more sensitive than tests for BSE infectivity in mice. Note also, as detailed at several points elsewhere in this book, that other more definitely documented microbial hazards may be associated with the eating of inadequately cooked meat products or with contaminated processed foods.

Specified Risk Materials. The SSC of the EU listed specified risk materials and adopted the following opinion in January 1998 as a guide to categorizing the potential infectivity of the tissues of BSE-infected animals (essentially cattle, sheep, and goats). Organs associated with high infectivity or with a risk of significant contamination during slaughter (category 1) include brain, eyes, spinal cord, dorsal root ganglia, dura mater, pituitary, skull (entire head excluding tongue) and vertebral column, lungs, and ovine and caprine spleens. Organs in the medium-infectivity category 2 include the total intestine from duodenum to rectum, tonsils, bovine spleen, placenta, uterus and fetal tissue, cerebrospinal fluid, and lymph nodes. Organs with low potential infectivity (Category 3) include liver, pancreas, thymus, bone marrow, bones, nasal mucosa, and peripheral nerves. Category 4 (no detected infectivity) is large and includes skeletal muscle, milk, blood clot, and serum. This line of reasoning was extended by the SSC to develop a suggested list of specified risk materials to be excluded from human and animal consumption except when derived from a BSE-free country with what they regarded as a negligible risk of BSE. In effect, the specified risk materials included virtually all of those in their categories 1 and 2. Many of the items carried an exemption up to the age of 12 months (ie, those tissues from young calves, sheep, and goats were not excluded, but ovine and caprine spleens and bovine, ovine, and caprine intestine were to be excluded at all ages). In practice, the heads of sheep and goats at all ages may have to be removed. In countries at high risk of BSE, it was considered probably appropriate to reduce the age limit for all of the specified risk materials from 12 to 6 months. And all tissues from cattle over 30 months of age might be considered as specified risk materials. The SSC opinion was that long bones from cattle under 30 months of age with no clinical signs of BSE may be considered as acceptable for human consumption.

57.10.2 Human Susceptibility to the BSE Agent

By mid-1998, when this chapter was finalized, 26 cases of nvCJD in the UK and 1 in France had been recognized and confirmed. All of the nvCJD cases examined to date are homozygous for methionine at codon 129 of the *PrP* gene (MM). The aminoacid 129 position is generally not polymorphic in animals; with few exceptions, animals are uniformly homozygous for methionine. In the general human population, MM homozygosity occurs in about 37%, valine homozygosity (VV) in 11%, and heterozygosity (MV) in about 51%. MM homozygosity is therefore thought to be a susceptibility factor for nvCJD; however, Zeidler and colleagues caution that this does not necessarily indicate that MV or VV individuals will be entirely resistant to nvCJD. It is possible, for example, that patients with these alternative genotypes might develop disease after a longer incubation period and perhaps with a different clinical presentation.[163] Incidentally, the MM genotype predominates (but not absolutely) among patients with sporadic and some forms of acquired CJD.

57.10.3 The Theoretical Pathogenesis of BSE in Man

It is likely that the patients who have so far developed nvCJD were severely challenged with the infective agent, possibly in their teenage years, or that some predisposing factor or factors operated in each case—but we remain uncertain. The families concerned have our greatest sympathy; they require much support, and the situation merits our constant concern. The likeliest time for such challenges to be delivered to man was when BSE-infected bovine products had free access to the human food chain (ie, in the mid-1980s and into the 1990s before the early control measures were effectively enforced). Perhaps the likely incubation period may be 5 to 10 years or more. Thus, a 14-year-old consuming an infective dose in 1986 might show early signs of nvCJD in the mid-1990s and would die 1 to 3 years later. The death of a young girl who had been a vegetarian for 11 years raises the possibility that some apparently nonanimal foodstuff or preparation may have contained a bovine ingredient or that the possible incubation period has to be measured in decades; moreover, the period of possible exposure to foodborne challenges with the BSE agent in the UK may extend back to the early 1980s or the 1970s.

Blood and Blood Products. In 1996, Dr Dealler[40] carefully reviewed his assessments of the risk of BSE transmission to humans from food, and he considered that human blood transfusion from a donor who had eaten BSE-infected food and was incubating the disease could be a hazard during the subclinical phase when the agent may be present in the blood (see commentary by Janet Morgan[106]). This speculation arises from evidence of transmission of other TSEs by blood and from the recent evidence of maternal transmission of BSE from cow to calf; and it presupposes bloodborne spread in that instance. Operskalski and Mosley pointed out in 1995[108] that hemophiliacs across the world have received large amounts of fresh blood, fresh-frozen plasma, and fibrinogen, and many have had "huge amounts" of clotting factor concentrates. There has been more than enough time for CJD to be seen in hemophiliacs if it is transmissible in this way, yet in three to four decades there has been not a single case of CJD recognized in a hemophiliac. In this context, we should be careful to discriminate between essentially cell-free blood products and whole blood that will contain white blood cells (leucocytes) as well as red blood cells. It is possible that the prions are carried in leucocytes; however, cell-free blood products not prepared from leuco-depleted blood will contain the contents of the cells. In 1994, CJD was diagnosed in a long-time plasma donor in the US, and this led to plasma quarantine and other actions being taken in the US. I am not aware of any scientifically documented evidence that CJD ever has been transmitted to man by human blood, but CJD has been transmitted experimentally to animals from human blood.[96]

Collections of abnormal prion protein (PrP^{res}) have been detected in tonsil tissue of human patients with nvCJD[69] and in the appendix of a patient who later developed nvCJD.[138] If the detection of PrP^{res} by microscopy of immunostained tissue can be equated with potential infectivity, this raises paradoxically conflicting reactions:

1. There is the worrying possibility that the agent of nvCJD has a much wider potential for tissue infectivity in man than our earlier mouse tests with the BSE agent led us to anticipate. If nvCJD patients show accumulations of PrP^{res} in lymphoid tissues, we cannot rule out possible bloodborne transmission of nvCJD, perhaps within leucocytes in the blood, at some stage in the development of the disease. Dr Dealler's warning is endorsed. Increasing anxiety regarding the possible infectivity of bloodborne cells and other tissues in human cases of nvCJD and the present nonavailability of a test to determine preclinical cases have led to restrictions on UK-derived human blood products, lest blood donors incubating undetected nvCJD (or sporadic CJD, for that matter, though sporadic CJD is not known to have such a wide tissue distribution) might have contaminated a preparation or a batch of material destined for human use.[77] The British government began to take action on blood products in this context in 1996[106] and in 1997 to 1998.
2. There is the hope that tests on human tonsil, appendix, and other lymphoid tissues may shed light on the pathogenesis of nvCJD in man. This could be linked with the ultimate goal of developing a blood-based test for the early diagnosis of CJD and nvCJD in man. It is to be hoped that parallel research on nonhuman primates may provide valuable clues.

The Oral Route. Let us concentrate now more specifically on the possibility of the oral route of transmission (a possible foodborne hazard) from cow to man, though surgically implanted or injected materials of bovine origin also could be candidate vehicles of infection with BSE.[142] About 1 gram of highly infective cattle brain is considered to be sufficient to produce BSE in a cow by the oral route. The ingestion of a quantity of infected bovine brain or a product containing this could have delivered a challenge of prions into the human alimentary tract before our control measures were enforced. The heat-resistance of the prions would protect against cooking temperatures, although there could be substantial reduction of infectivity.[135,136] The proteinase resistance of prions might protect against proteolytic digestion in the gut (not yet formally proved).

The prions might be taken up by the lymphoreticular system via M cells in Peyer's patches throughout the gut. (It is even possible that they could be taken up by lymphoid tissues in the oropharynx.) Perhaps host clearance mechanisms might be crucially compromised on occasion by another infecting agent such as that of infectious mononucleosis, or measles, or influenza. The prions then might transform normal host PrP to boost their numbers in the local cells and to build up the infection in organs such as the spleen and other lymphoreticular tissues. Hill and colleagues have shown how abnormal PrP accumulates in the lymphoid tissue of the tonsil in nvCJD,[69] and we now know that the appendix may be involved. Kimberlin and Walker's studies of the dynamics of replication of the sheep scrapie agent indicate that agent replication within the nervous system and not the lymphoreticular system is a key factor,[84] and Blättler and colleagues have shown, in studies of scrapie in mice, that a continuous chain of PrP-expressing tissue is needed for the prions to spread from peripheral sites to the CNS.[11] Colonization of the lymphatic system is probably necessary for neuroinvasion, but further PrP^{res} expression, possibly in the peripheral nervous system, seems to be necessary for successful CNS involvement. PrP is particularly abundant in neurones, and Aguzzi postulates a "neuro-immune connection" in the spread of prions in the body.[2] We can theorize by analogy with the neurotropism of poliomyelitis from the gut, or of rabies virus, or tetanus toxin from a wound. By tracking along local splanchnic (intestinal) nerves or other peripheral nerves to the spinal cord, the prions would then gain access to the brain where, apparently, the production of abnormal PrP is up-regulated and neurological function is impaired progressively.

57.10.4 Surveillance Work: Past, Present, and Future

The epidemiology of CJD and nvCJD in the UK is being monitored carefully by Dr R.G. Will and his team at Edinburgh.[118] Important studies that have sought to identify risk factors for sporadic CJD in the past include those of Bobowick et al. in 1973,[12] Masters et al. in 1979,[99] Kondo and Kuroiwa in 1982,[89] Will et al. in 1986,[151] Davanipour et al. in 1985 and 1987,[35,36] Brown et al. in 1987,[15] and Harries-Jones et al. in 1988.[64] And we add much recent speculation, summarized in this chapter, on possible risk factors for nvCJD.

What must now be done? Further careful clinical, pathological, epidemiological, and microbiological studies of sporadic and other CJD cases (for example, studies by Brown and colleagues[15,16]) will give answers if the present level of international cooperation and liaison is sustained into the forthcoming century. The continuation of the UK surveillance program is vital and it must be ensured for several decades while it adapts to changing circumstances.[149,151,152] The discovery that the agents of nvCJD and BSE are identical is accepted as proof that the agent of BSE has been transmitted to man. The annual numbers of deaths from various forms of CJD recorded in Britain are given in Table 57–7. In 1990, when accurate data began to be available and hospital referral of any suspected case was recorded, there were 33 cases. The numbers increased thereafter, perhaps partly due to improved surveillance and awareness; from 1991 to 1998, annual totals of 36, 51, 46, 59, 47, 60, 79, and 72 until the end of 1998 are a cause for some anxiety, and the contribution of nvCJD cases (13 in 1995–1996, 10 in 1997, and 16 in 1998) clearly fuels our anxiety. There is an inevitable delay in getting true figures for the current year because investigations are still in progress. Several workers believe that there is still under-reporting because of missed or misdiagnosed cases of sporadic CJD.[149] New diagnostic tests for CJD are needed urgently.[88] The production of a cerebrospinal fluid test for human and animal transmissible spongiform encephalopathies is a promising step forward,[75,93,153,164] and the preparation of monoclonal antibodies specific for PrP^{res}[87,105] is most encouraging, while we anxiously await further developments with the new variant disease.

Of course, continuing surveillance of BSE and scrapie in cattle and sheep in the UK is essential, and this must be actively extended worldwide. There are worrying suggestions in the news media and elsewhere that possible cases of BSE in cattle are being missed in Europe and the US, that information may be suppressed in some countries, or that mismanagement of feedstuffs abroad may have disastrous consequences.[14,117] The need for increased vigilance and rigor surely must be obvious. An official Inquiry was set up in London under the chairmanship of Lord

Table 57–7 Annual Numbers of Deaths from CJD in the UK (1985–1998)

		Deaths of Definite and Probable Cases of CJD in UK					
Year	*Referrals*	*Sporadic*	*Iatrogenic*	*Familial*	*GSS*	*nvCJD*	*Total*
1985	—	26	1	1	0	—	28
1986	—	26	0	0	0	—	26
1987	—	23	0	0	1	—	24
1988	—	22	1	1	0	—	24
1989	—	28	2	2	0	—	32
1990	53	28	5	0	0	—	33
1991	75	32	1	3	0	—	36
1992	96	43	2	5	1	—	51
1993	78	38	4	2	2	—	46
1994	116	51	1	4	3	—	59
1995	87	35	4	2	3	3	47
1996	134	40	4	2	4	10	60
1997	161	58	6	4	1	10	79
1998	150	50	3	3	0	16	72

Total number of definite and probable cases of nvCJD to end of 1998 = 39 UK patients.

Source: from Department of Health Press Release, August 3, 1998, and CJD Surveillance Unit, UK.

Justice Phillips in 1998 and a report of its very detailed deliberations is anticipated in mid-2000.

57.11 CONCLUDING REMARKS

On present evidence, there is understandable concern that many of the UK population will have eaten processed meat products incorporating potentially BSE-infected bovine tissues—such as brain and other offals banned since 1989—at some time in the 1980s, while doubt remained about the rigor of our enforcement procedures in the UK even up to 1995. Calf brain was not banned when this chapter was finalized in late 1998; there is no persuasive scientific evidence that its consumption is hazardous, but the sensitivity of mouse bioassays used to detect BSE infectivity is not absolute. Inadvertent parenteral experiments on the possible transmissibility of BSE to man that might have taken place in the 1980s must include the injection or implantation of bovine-derived materials that were in common medical and surgical use in Britain and across the world in the 1970s and 1980s,[10,142,148] but we have no evidence to incriminate injections or implantations of bovine-derived materials or of substances associated with bovine products in pharmaceutical or cosmetic preparations.

The recognition of 39 British cases of nvCJD, plus one in France, is most worrying. Studies reported in October 1997 show that nvCJD is distinct from other forms of CJD and they provide convincing evidence that the agents of nvCJD and BSE are the same. Moreover, the agents recovered from cats with FSE in Britain and from captive exotic bovids that went down with BSE in British zoos had the same profile as agents recovered from cattle with BSE.

Diringer's belief in the wider infective potential of the BSE agent is sustained, but the debated link with scrapie remains to be resolved. Note, however, that the CJD illnesses in our farmers that gave rise to much recent speculation did not have the characteristic clinical features of nvCJD, and the agent recovered from such cases does not have the profile of the BSE agent. Is it possible that sporadic CJD has some other relationship with animal prion diseases that we do not yet recognize, or might it be that the BSE agent is only one of several prion agents that might pass directly or indirectly from animals to man? We do not know.

There is much in biomedical science that is uncertain. The transmissible encephalopathies reveal the facts slowly while some impatient observers press for draconian measures and criticize the steps that have been taken by a responsible and well-informed administration in Britain, advised by scientists of world class who have to work with great care for the safety of themselves and others.[1] Several scientists, frustrated by apparent bureaucratic inertia, have joined the opposition,[41–43,80,91] and we should have paid more attention to some of their views, but many of the critics have not acknowledged the complexity of the problems and the caliber of the scientists and administrators involved. It is a blessing that the pioneer work on scrapie was done in Britain by Gordon[60] and Greig[62] and Wilson and Stamp and others,[131,157] and that continuity was ensured by the work of scientists such as Dickinson, Fraser, Hadlow, and Kimberlin[46,47,84] and by Hope and the neuropathogenesis group at Edinburgh.[76] Meanwhile, the splendid investigations undertaken in the US and elsewhere by Gajdusek and his teams[12,15,17,56,58,59] gave us insight into the human model of kuru and beyond, and the brilliant persistence of Prusiner and his colleagues in developing the concept of prions[112–115] complemented our growing awareness of new mechanisms of disease. Prion theory continues to be challenged (see Manuelidis,[97] Alper,[6] Finkel,[54] and McCarthy[95]), but it has come a long way. At the same time, investigators recruited by, or collaborating with, Matthews,[33] Collinge,[29,30] Brown,[15–18] and Will[18,150–153] and others kept us in touch with the expanding story of CJD in man. The remarkable work done by Wilesmith and Bradley and their groups[13,144–147] after the discovery of BSE in cows by Wells and his coworkers[140] ensured that the UK authorities have continued to have all of the facts on BSE as promptly and as accurately as possible, despite the complexity of investigations in this field. And we owe a debt to the Southwood team for their prompt and accurate conclusions based on the evidence available at the time.[119] The continuing care of workers such as Kimberlin,[83] Tyrrell,[130] Taylor,[133–136] Fraser,[55] Bruce,[19,20] Almond,[4] Pattison,[5,109] and many others is admirable. It is essential that the necessary resources should be provided to ensure that the vital surveillance of BSE and CJD and scrapie and the associated research and advisory services are sustained over the next two decades. It is also imperative that present control measures and any further precautionary orders be rigorously enforced in the UK and abroad.

The author of a chapter in a scientific textbook cannot expect to be absolutely up-to-date even in the year of publication. The facts of BSE as we presently understand them allow us to hope in the late 1990s that the worst of the bovine outbreak is over, as far as British cattle stocks are concerned. There are some worries about the control of BSE abroad and possibly in UK sheep.[27] The outlook for man is uncertain. The avenue of infection was almost certainly the oral route. It is most unlikely that cow's milk could be the vehicle, and there is no evidence that (unprocessed) prime beef could have transmitted BSE to man. The supplementary precautions introduced in the mid-1990s add to this assurance. The likely vehicles of infection for man were various bovine products associated with significant BSE infectivity—infected bovine brain and other offals before they were banned in 1989 and before the ban was rigorously imposed and made fully effective in the mid-1990s when other loopholes were closed; for example, mechanically recovered meat from sites such as the vertebral column was relatively recently regarded and controlled as another probable vehicle. We cannot yet entirely exclude other species or vehicles from possible involvement. The number of human patients known to be affected with nvCJD by March 1, 1999 is 40 in the UK plus 1 case in France. Sadly, we must anticipate more human cases. In early 1997, senior and well-informed biomedical scientists made tentative predictions based on various assumptions. On this occasion, they were reported responsibly. If the numbers of patients affected remain low at less than 20 per year for the next few years, the total number of human cases may be 100 or fewer. But if there is an annual doubling or tripling of new cases in the years after 1996, there could be hundreds or thousands of cases. And a worse-case scenario of tens of thousands of cases was envisaged if extended incubation periods of 20 or 30 years occurred. By early 1999, we have seen 40 cases in a population of about 55 million and we think that the incubation periods have been of the order of 5 to 12 years. We must expect to see more evidence of the agent's potential to cause human disease within the next decade at least. There are substantial grounds for the belief that BSE is not readily transmissible to man, but we must wait a decade or two with "substantial uncertainty" into the forthcoming century for the information that might endorse or challenge this optimism.

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Note: Up-to-date information on aspects of BSE in relation to food is made available by the Institute of Food Science and Technology, IFST (UK), on the World Wide Web. Access via the IFST homepage is obtained by use of the URL http://www.easynet.co.uk//ifst.

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IV Assurance of the Microbiological Safety and Quality of Food

58

Good Manufacturing Practice, HACCP, and Quality Systems

Jean-Louis Jouve

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58.1 INTRODUCTION

The production, distribution, and marketing of foods with good microbiological quality and safety require control to be exercised over three different categories of microorganisms: Those that have the potential to cause foodborne diseases, those that cause food spoilage, and those that grow and produce desirable changes. Although extensive control procedures are needed to manufacture fermented products, such procedures will not be addressed in this chapter, which will instead focus on the control of pathogens and spoilage microorganisms; for information on fermented products, see Chapters 19, 23, and 26.

Like much of microbiology, the general strategy for the control of pathogens and spoilage microorganisms goes back to Louis Pasteur and his work in the 1860s. This strategy is basically one of exclusion, which requires reducing or eliminating the initial microbial load, preventing or minimizing further contamination (or recontamination), and, where necessary and feasible, preventing or delaying their subsequent growth in a food. The responsibility for developing and implementing this strategy belongs to all those involved in the food chain (ie, plough to plate); each has to focus on the ways of most effectively and efficiently ensuring that the procedures they apply will lead to the production of a safe and wholesome product.

For many years food producers, processors, retailers, and regulators relied on the use of Good Manufacturing Practice (GMP), control of hygiene, and microbiological testing of end-products to produce foods that are microbiologically safe and stable. As concern about the microbiological safety and quality of foods has increased, the shortcomings of this traditional approach have become recognized.[24,30,50,65] In recent years, therefore, this approach has been strengthened by the use of the Hazard Analysis Critical Control Point (HACCP) system and application of the concepts of quality assurance and quality management.

The aim of this chapter is to outline the way in which GMP, HACCP, and quality systems can be used in an integrated and effective way to ensure the production of microbiologically safe and stable foods.

The author wishes to thank Tony Baird-Parker and Tony Mayes for their useful suggestions and assistance in developing this chapter.

58.2 AN INTEGRATED APPROACH TO CONTROL MICROBIOLOGICAL QUALITY AND SAFETY

Food should be safe with respect to microorganisms capable of causing foodborne diseases and remain wholesome to the end of its shelf life. Absolute product safety (ie, freedom from all microbiological risks) is not achievable. The problem that regulatory authorities and food companies must face is to reduce such risks to acceptable and predictable levels, taking into account the cost-benefit implications involved in their control.

Two procedures can be used in tandem to identify the microbiological risks of concern in foodstuffs and what specific measures need to be applied for their control. The first, which is principally the function of the regulatory authorities, consists of applying a formal risk assessment procedure to determine whether a microbiological hazard, or a group of hazards, is of significant concern in foods consumed by a designated population and what measures should be generally applied for its control.[63] The methodology and use of risk assessment for this purpose are discussed in detail in Chapter 65. The second, which will be discussed in this chapter, will ideally follow from the recommendations of the first procedure, and is the development and application by the food industry of specific procedures to control microbiological hazards at their source, recognizing that it is the responsibility of industry to ensure—as far as is reasonably possible—that products offered for sale are safe.

The management of a food company is responsible for establishing a product safety policy, for defining the objectives to meet this policy, and for implementing these through appropriate programs. In addition to establishing means for adequate control and assurance of the safety of their products, management should provide means for assessing the performance of programs to ensure safety and to identify means for increasing their effectiveness and efficiency.

Design is crucial, and safety should be adopted as a basic design parameter. Designing for microbiological safety encompasses not only the product, but also the process and its components, such as the factory, equipment, people, and any specific treatment used to eliminate or minimize significant microbiological concerns. Manufacturing is a key function. At all stages, from procurement of raw materials and ingredients to final product dispatch, care must be taken to control all factors and activities that affect product safety. Distribution and sale also play a significant part through appropriate labelling (such as storage and use information to distributors and consumers). Observation of consumer practices and investigation of complaints also provide data to improve product and process design.

Setting apart managerial practices, which are far beyond the scope of this chapter, implementing control at the source to manufacture safe and wholesome food requires appropriate tools to be used in an integrated manner (Figure 58–1). This figure shows how GMP, HACCP, and quality management systems can be used to build a structured approach to the production of safe and high-quality foods.

GMP documents, and specifically the hygiene requirements they contain, describe and define the basic conditions for the hygienic production of a foodstuff. These usually will include requirements for: the hygienic design, construction, and operation of the manufacturing plant; the hygienic construction and use of food processing equipment; scheduled maintenance and cleaning of equipment and facilities; and personnel training and their hygiene requirements. Details of these requirements will be found in Chapter 59 and Section 58.3.

HACCP principles have gained worldwide acceptance as the most effective means of ensuring that foods are safe to eat.[16] The HACCP system is a structured approach to hazard management that aims to prevent product safety problems by identifying those hazards (biological, chemical, or physical agents) associated with the production, distribution, and sale of a particular foodstuff and means for their control to safe levels. Use of HACCP results in a safety assurance plan and system specific to a given product, its process of production, and the hazards involved. It can only be applied to a production operation applying GMP and cannot replace the need to apply GMP requirements. A detailed discussion and description of the use of HACCP will be found in Section 58.4.

A company producing a foodstuff must organize itself in such a way that all factors that affect product features and characteristics, including technical, administrative, and human factors, are under control. Such control should be aimed at the prevention of problems and toward their elimination or reduction. Such an organizational approach is broader in scope than product safety, and encompasses all quality attributes. The companywide quality approach is developed appropriately and implemented through a quality system that covers the organizational structure, responsibilities, procedures, processes, and re-

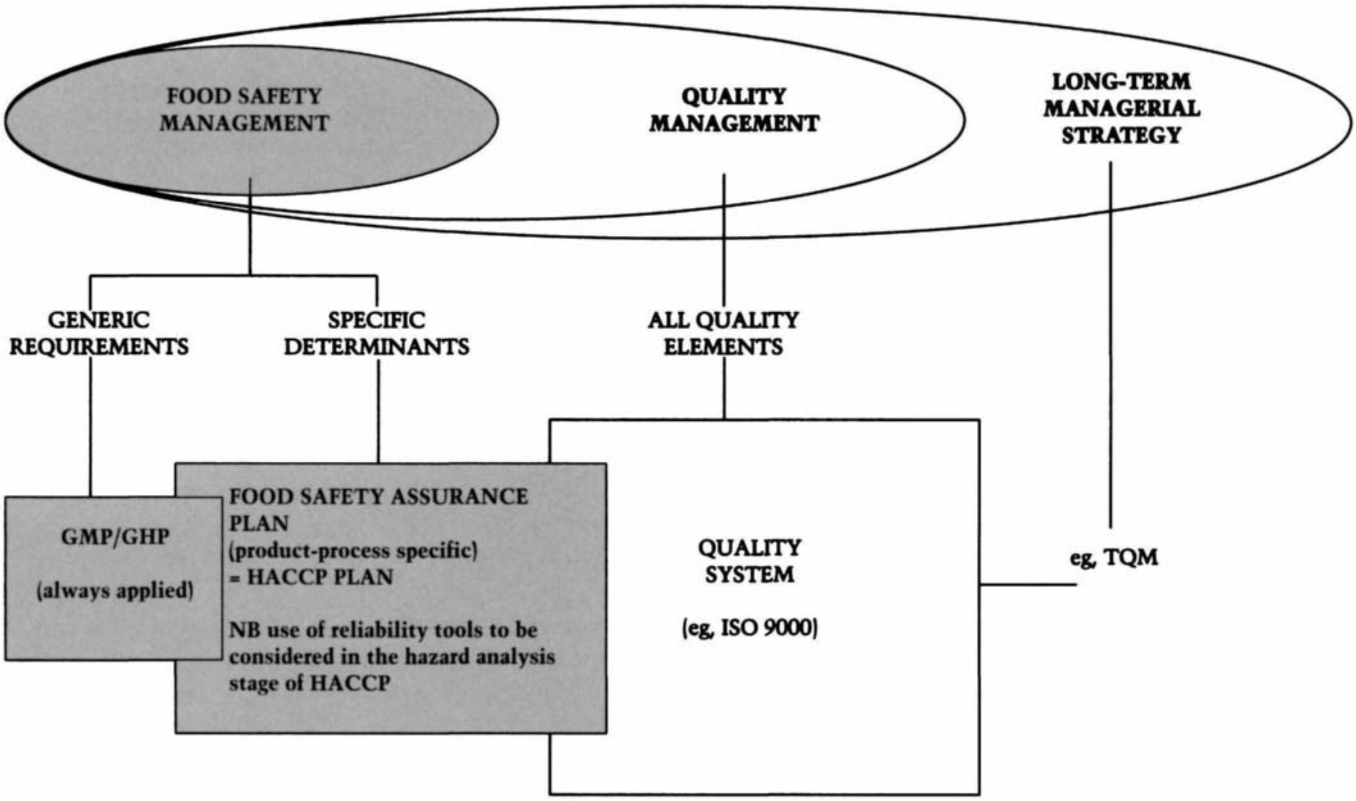

Figure 58–1 An Integrated Approach To Control the Microbiological Safety and Quality of Foods. *Source:* Reprinted with permission from ILSI Europe, *Food Safety Management Tools*, © 1998, International Life Science Institute.

sources needed to implement the overall management of quality. A quality system applies to, and interacts with, all phases of a product cycle, from marketing and design, through procurement and production, to distribution and sale. Specific guidance tools have been designed to aid food manufacturers in establishing such quality systems, among which the ISO 9000 series of Quality Management and Quality Assurance Standards have achieved worldwide recognition and acceptance.[33–37]

Within the framework of a quality system, the specific practices related to a given product and attribute (eg, safety) are specifically addressed in a specific plan (eg, a product safety assurance plan). Such a specific safety assurance plan is an intimate part of the quality system but can be used separately, in contractual or regulatory situations, to provide evidence of adherence to specific expectations or requirements (see Section 58.5 for details).

Finally, for achieving a permanent improvement in company performance, including safety as a key parameter, a more cultural and less hierarchical approach to management, based on the participation of all members of an organization, is to be recommended. This is the Total Quality Management (TQM) approach, which complements the approaches previously mentioned. It is important for the long-term success of a business, giving, through greater commitment and motivation of the total work force, better customer satisfaction. Further information on TQM can be found in the literature.[16,18,39,49,56]

While ideally every operator would benefit from the combined strengths of applying GMP, HACCP, and a quality system, practical implementation needs to be flexible and to be only as comprehensive as necessary. Two factors should govern these: the level of safety assurance required, which directs the certainty of controls needed, and the complexity of products produced and the production process.

GMP, particularly basic hygiene requirements, should be applied always. For low-risk products manufactured or processed using traditional procedures, the application of GMP usually will provide adequate security; an HACCP plan is not likely to be necessary or needs only to be of limited extent, un-

Exhibit 58–1 Example of the Format of a Good Manufacturing Practice Document

1. **General Provisions**
    - 1.1 Scope
    - 1.2 Definitions
2. **Personnel**
    - (a) Health status and disease control
    - (b) Cleanliness
    - (c) Education and training
    - (d) Supervision
3. **Buildings and Facilities**
    - 3.1 Plant and grounds
        - (a) Location and grounds
        - (b) Plant construction and design
    - 3.2 Sanitary operations
        - (a) General maintenance
        - (b) Substances used in cleaning and disinfection and their storage
        - (c) Pest control
        - (d) Sanitation of food contact surfaces
        - (e) Storage and handling of cleaned portable equipment and utensils
    - 3.3 Sanitary facilities and controls
        - (a) Water supply
        - (b) Plumbing
        - (c) Sewage disposal
        - (d) Toilet facilities
        - (e) Hand-washing facilities
        - (f) Rubbish and waste disposal
4. **Equipment**
    - 4.1 Design of equipment and utensils
    - 4.2 Maintenance of equipment and utensils
5. **Production and Process Controls**
    - 5.1 Processes and controls
        - (a) Raw materials and other ingredients
        - (b) Manufacturing operations
    - 5.2 Warehousing and distribution
6. **Documentation and Records**

Source: Adapted from Current Good Manufacturing Practice in Manufacturing, Packaging or Holding Human Food, *Code of Federal Regulations 1990,* Title 21, Part 110, U.S. Government Printing Office.

less required by law. However, for microbiologically sensitive products such as low-acid canned foods, chill-stored cooked meats, or complex operations involving a number of stages, and where risks of microbiological contamination are high, a detailed safety assurance plan based on HACCP is necessary to focus on those requirements critical for product safety.

Where the complexity of an organization increases with several levels of responsibilities among staff and where a number of departments contribute to the production of a food, a top-down structured approach to ensuring that safety requirements are met is necessary. Thus such a company should have a quality system in place to provide evidence that it is properly organized to control all stages and activities that affect product quality and safety.

58.3 APPLICATION OF GOOD MANUFACTURING PRACTICE

58.3.1 General Concepts

GMP defines and documents all requirements (premises, equipment, and operating practices) necessary for the production of food of acceptable quality.[28] With respect to the microbiological safety and quality requirements of such a food, it specifies the basic hygiene measures that all establishments covered by the scope of a GMP should meet. As was mentioned previously, these basic hygiene measures are a prerequisite to the application of HACCP, allowing HACCP to focus on steps, practices, or procedures critical for the safe processing or handling of a food in a particular establishment (see Section 58.4).

GMP documents in the form of regulations have been developed by regulatory authorities (eg, the US regulations on GMP),[10] the format of which is shown in Exhibit 58–1.

Other documents have been developed in the form of codes of hygienic practice by international organizations such as the Food Hygiene Committee of the Food and Agriculture Organization/World Health Organization (WHO) Codex Alimentarius Commission.[11] In addition, a large number of good manufacturing and hygiene codes have been issued by the food manufacturing and processing industries, often in cooperation with other groups and regulatory bodies, and on a national or international basis.[27] Such codes, although usually advisory, provide a reference point for both the guidance of manufacturers and enforcement authorities. Some are specific codes containing requirements for production of a specific foodstuff, whereas some are general codes covering the principles common to the production of the majority of foods. The latter may be complemented by specific codes for the production of specific types of foods. Thus the European Union Directive on the Hygiene of Foodstuffs (93/43/EEC) mandates basic hygiene requirements for food production, distribution, and sale but leaves industry to document any additional and specific hygiene requirements in the form of nonmandatory guides.

Generally, GMP documents address the following activities: the hygienic design and construction of food production premises; the hygienic design, construction, and use of food processing equipment; cleaning and disinfection of food premises and equipment; selection of raw materials and production conditions; training and hygiene of personnel; and appropriate documentation.[11,55] These activities are discussed in the following paragraphs and considered in more detail, together with specific examples of their application, in Chapter 59.

58.3.2 Hygienic Design and Construction of Food Manufacturing Premises

Appropriate design and construction of premises are necessary to limit entry, multiplication, and spread of microorganisms in the environment where a food is being produced or manufactured and, most importantly, to prevent or minimize cross-contamination of the products. Important aspects to be considered include the following:

Control of Conditions External to the Facility. To minimize the entry of contaminants into the plant, surfaces used for foot and vehicle traffic should be paved; covered containers should be used for rubbish; weeds and rubbish should be regularly removed; and unwanted equipment stored away from production areas.

Rooms Should Be Arranged To Ensure Straight-Through Flow of Products from Raw Materials to Final Product. Areas where potentially contaminated raw materials or other materials are handled should be adequately separated from areas where final product is handled. In "high-risk" areas, where prevention of cross-contamination is crucial, segregation also should be extended to staff working in them and any fixed or mobile equipment used.

Durability, Integrity, and Cleanability of Surfaces of Structural Components of Buildings and Facilities (Floors, Walls, and Ceilings). Materials used should be durable, impervious, nonporous, easy-to-clean (particular attention should be paid to avoid parts that are difficult to clean such as corners at junction between floor and walls) and free from cracks and crevices. Occupational safety considerations must not be overlooked (eg, nonslip floors). All surfaces must be well maintained and readily cleaned.

Control of the Environment. Air flow should be directed from clean to dirty products to minimize the spread of microbial contaminants by air. Filters, ducts, and other plant use of air conditioning should be inspected frequently, cleaned, and maintained properly. Temperature should be controlled to avoid major variations in room temperature because large differences of temperature between air and product may result in condensation and a source of microbial contamination.

Water Supply and Drainage Systems. Water used in processing should be both potable and of good microbiological quality. An ample and suitably located supply should be provided at the temperature necessary for use. To prevent contamination, cross-connection of plumbing with nonpotable water supply should be eliminated and back-flow prevention devices installed wherever back-flow or back-siphonage may occur. Special care should be taken in the design of effluent disposal systems that should be constructed and serviced so that the flow is always directed away from the production areas, with no possibility of back-flow or seepage.

Pest Control. Rodents, insects, birds, or other animals can be carriers of spoilage or pathogenic microorganisms. Measures for pest control include:

- elimination of breeding places and natural habitats in the surroundings of the plant such as weeds, brush, tall grass, litter, waste or refuse, and stagnant water
- actively excluding or restricting access to food storage and processing areas by using screens on windows, air curtains, or automatic closing devices on doors and sealing cracks, crevices, and holes
- and setting up extermination procedures (elimination of nesting sites, installing electrocution devices for insects, and the use of chemicals or biological treatments).[58] The outside of the facility should be inspected regularly for new places where pests could enter.

Appropriate Design, Location, and Maintenance of Sanitary Facilities To Minimize Potential Sources of Contamination. These include water supply and distribution systems, handwashing facilities, clothes changing areas, sewage disposal, and the storage and removal of waste.

58.3.3 Hygienic Design and Construction of Food Processing Equipment

When selecting food processing equipment, consideration is often given only to requirements such as suitability to perform the intended function, size needed for the production rate, reliability, ease of operation and technical maintenance, compatibility with other equipment, safety of operators, and cost. Too often, little consideration is given to hygienic design aspects of equipment; poor design and construction can lead to microbiological contamination of products. The main identified factor causing unsatisfactory microbiological performance is poor cleanability that may result from unsuitable construction materials; poor design of specific parts such as injectors, gaskets, glands, valves, or pumps; inadequate rounded corners, rolled edges, sharp angles, and poor welds; and existence of void spaces and dead legs inaccessible to cleaning fluids (eg, in clean-in-place [CIP] systems). Information on the correct design of such processing equipment and components will be found in Chapter 59.

Other conditions allowing microbial build-up and product contamination may result from the inability to drain equipment properly, poor accessibility to the equipment itself or to specific parts or surfaces in direct contact with food, poor repair, maintenance, and improper use of equipment.

58.3.4 Cleaning and Disinfection of Food Premises and Equipment

A regular cleaning and disinfection program is crucial to plant and food hygiene. Such a program should be treated as an integral part of the production process and "not regarded as an end-of-shift chore, liable to be hurried or superficial";[1] it should include both product contact surfaces and associated areas such as walls, floors, and ceilings.

Sanitary operations in food processing have two purposes. The first is cleaning to remove food residues that provide the nutrients necessary for microbial growth and may prevent proper functioning of equipment. It also removes the majority of microbes through the physical action of washing and rinsing. The second is disinfection (or sanitization) intended to reduce the microbial population that adhere and survive cleaning to a level where they will not be a significant source of microbial contamination of product coming into contact with the equipment. After cleaning and disinfection, the areas must be protected from recontamination before use.

Wet cleaning involves:

1. Selection of a suitable system (high pressure low volume; low pressure high volume; CIP systems, manual, foam/gel) depending on the design of the equipment or area to be cleaned, the soil type (tenacious or water soluble), the soil level, the design features, materials of construction, and operational characteristics of the equipment and conditions within the facility.
2. Selection of chemicals (detergents and disinfectants) taking into account properties such as the spectrum of antimicrobial activity (Gram-positive bacteria, Gram-negative bacteria, spores, bacteriophages), corrosiveness, irritability to skin, performance in hard water, presence of organic matter, incompatibility with equipment or other cleaning and disinfecting agents, stability of use solution, stability of hot solution, effectiveness at use pH, whether leaving active residues, maximum level permitted by law, and cost.[13,14]
3. Determination of an appropriate cleaning procedure or cycle including brushing, scraping, prerinsing, immersion in detergent, rinsing, disinfection, drying step, or a combination of these. The specific aims of these procedures are to control four basic factors (ie, mechanical force, concentration of chemicals used, temperature, and contact time). It should be kept in mind that with the exception of specific products, such as combined detergent-disinfectant products (detergent sanitizers), most disinfectants are ineffective in the presence of food residues.

Dry cleaning of a plant used for the processing of dry foods or ingredients presents special problems because the equipment is not usually designed for wet cleaning; moisture allows dry foods to support microbial growth and/or result in the formation of difficult-to-remove deposits. In dry processes, cleaning usually involves mechanical action (scraping or brushing), use of vacuum cleaners, and, where necessary, disinfection with ethanol (70%), which vaporizes quickly and leaves the surface of the equipment dry. The possible use of carbon dioxide freezing and acoustic devices is under consideration for dry cleaning but is generally too costly for general use.

Detailed technical information on cleaning equipment, chemical agents, and their use can be found in a number of publications.[19,21,24,29,43,57] (See also Chapter 59.)

In the framework of GMPs, the management of any food establishment is responsible for maintaining an appropriate cleaning and disinfection program. The following aspects should be considered:[22]

Selection of Type of Cleaning Staff. This may include: hiring the services of staff from a specialized company; using its own cleaning staff; cleaning by production personnel; or a combination of these such as production personnel beginning the cleaning process before leaving the work area and handing over to separate cleaning staff to complete the task.

Providing Services and Facilities. Services and facilities include the following: appropriate management of the cleaning staff (training in appropriate procedures, adequate supervision, stability, wages, acknowledgment of job well-done, and incentive for better performance); sufficient and well-placed hot water or steam outlets; appropriate time to clean and disinfect the equipment properly; adequate design of cleaning equipment (eg, for CIP [flow rate of cleaning fluid], for hard surfaces [cleaning jet design and appropriate temperature and pressure of water]); appropriate design, construction, installation, and maintenance of production plant (see Section 58.3.2); and selecting the appropriate cleaning system and related chemicals.

Providing Appropriate Means to Monitor and Verify the Effectiveness of Cleaning and Disinfection Procedures. This could include swabbing surfaces of equipment after cleaning/disinfection and culturing the residual organisms or using an indirect measure of cleanliness such as measurement of adenosine triphosphate (ATP); testing of food samples produced during the start-up of production; and environmental sampling.[15,24]

Development of Written Instructions. Such instructions should outline the following: cleaning and disinfection procedures; frequency of cleaning; equipment and chemicals needed; quantities of chemical to be used, method for preparation of the required dilutions, and precautions for handling the agents; person responsible for hygiene and monitoring (where direct responsibility is delegated to subordinates, it should be stated clearly who is held responsible); and methods for monitoring the effectiveness of cleaning and disinfection regime. Certain areas or equipment for food preparation may require particular attention because they are critical to the microbial safety or quality of the food being prepared. Such specific information should be included in the sanitation procedures.

Improving Hygiene. This should be considered when: food has been implicated in a foodborne disease outbreak; when stored samples spoil before the expiration date or when a number of spoiled food items are returned by customers; changes in distribution occur requiring longer shelf life; and products do not comply with microbiological criteria established by the producer, customer, or regulatory agency. However, before carrying out hygiene improvements, it is important to consider whether these problems may not have resulted from deficiencies in general hygiene, but from production deficiencies such as times and temperatures used for cooking or cooling or poor storage of raw materials or end-products.[24] Records always should be kept of cleaning and disinfection activities and of results of any tests of their effectiveness.

58.3.5 Selection of Raw Materials and Production Conditions

All operations, from receiving raw materials and ingredients through preparing and processing, to packaging, storage, transport, and distribution, should be conducted in an hygienic way. All reasonable precautions should be taken to ensure that production procedures do not contribute microbiological (and other chemical or physical) hazards to the processed product. Specific consideration should be given to the following:

Starting Materials (Raw Materials and Ingredients). Food manufacturers must be aware of the potential microbiological hazards in starting materials and prepare purchasing specifications accordingly. Such specifications should take into account the microbiological status of the incoming materials, the product's formulation, the process to be applied, and the intended method of distribution and use. Specifications, agreed on between manufacturer and suppliers, are a safeguard for all parties concerned. Compliance with these may be verified by a variety of means, including purchasing the materials under a supplier's guarantee or certification or examination of these materials for microbiological contamination; the use of microbiological criteria in purchasing specifications is discussed in Chapter 65. Raw materials should be inspected and segregated as necessary to ensure they are clean, wholesome, and fit for processing. They should be appropriately handled, disposed of, and stored under good conditions with an effective stock rotation system. Containers and carriers should be inspected on receipt to ensure that their condition has not contributed to the contamination or deterioration of the product. When ice is used in contact with food products, it should be made from potable water and protected from further contamination.

Processing Steps. All food processing steps, including packaging and storage, should be conducted under conditions necessary to minimize the potential for unacceptable survival or growth of micro-organisms or for the cross-contamination or deterioration of the food. This requires the application of suitable processes and procedures to limit microbial growth (eg, chilling, freezing, curing, drying, conserving, fermenting, use of preservatives [see Chapters 5 to 10]) or to kill microorganisms (eg, cooking, pasteurization, sterilization [see Chapters 3 and 4]), and to prevent subsequent contamination (eg, packaging, appropriate warehousing, and storage). Control of such processes and procedures is achieved by monitoring physical factors such as process time/temperature relationships, humidity, a_w, pH, pressure, and product flow rate. Also specific key manufacturing processing operations that may affect the microbiology of the product (eg, heat processing, dehydration, freezing, refrigeration) should be monitored and controlled to ensure that mechanical breakdown, time delays, temperature fluctuations, and other factors do not contribute to contamination or deterioration of the processed product.

Packaging Materials. These should not transmit contaminants and objectionable substances to the products, should conform to regulations and specifications, and should provide adequate protection from contamination. Coding of products should be used, together with appropriate records, to enable lot identification and to facilitate, where necessary, segregation of defective products. Warehousing and shipping conditions should prevent contamination and growth of microorganisms and protect against any undesirable deterioration of the container and the product.

58.3.6 Training and Hygiene of Personnel

Microbiological problems mostly result not from intentionally unsound practices but from ignorance, negligence, or both. It follows that manpower is a crucial factor. The most effective approach to training aims to establish a positive attitude to microbiological quality, product safety, and wholesomeness. Such an approach includes development of knowledge and skills through an appropriate training program. All groups of personnel should be involved, including managers, supervisors, and operators. Many helpful training materials are available from national or international organizations, research or manufacturers' associations, and consultants.[24,41,64] Involvement of the work force is important in hygiene and quality tasks at the production line by, for instance, workers' consultation (eg, hygiene or quality circles), transfer of knowledge, task design (or redesign), feedback of information, autocontrol, and individual commitment to implementation of hygiene management.[17] This also will motivate the staff to maintain or improve their personal hygiene including:

- general personal hygiene
- wearing appropriate clothing and changing and washing of clothes; not using tobacco, or eating, smoking, or chewing while working in a food handling area
- thoroughly washing hands and removing from hand all jewelry that cannot be adequately secured and sanitized
- use of disposable gloves, where appropriate, or washing and sanitizing reusable gloves where they are used
- wearing a suitable hair cover.

It is the responsibility of management to provide the appropriate facilities and instructions and to set an example of compliance with these requirements.

Pre-employment medical examination and periodic re-examination of food handlers for microorganisms likely to be transmitted by food is an established practice in some countries; however, a specialized working group of the WHO,[66] after reviewing health examinations of food handling personnel, concluded that routine examination of food handlers has severe limitations that considerably reduce its usefulness as a control measure. Such examinations, therefore, should have a very low priority and be only target oriented (eg, at persons directly involved in handling ready-to-eat chill stored foods prior to packaging). A more effective, and generally applicable strategy, is to educate food workers on hygienic food handling and to apply appropriate control of food hygiene (eg, by the application of GMP and HACCP).

58.3.7 Documentation

We have already stressed the importance of documentation; all GMP procedures, including sanitation, should be documented and appropriate records of performance maintained. Documentation reflects the commitment of the establishment's management to consistently apply the basic control measures identified in GMPs. Good documentation also demonstrates to third parties that the people responsible for the establishment know and understand their operations and, in particular, how to maintain good hygienic practices in their establishment.

Documentation in GMPs concerned with hygiene should consist, as a minimum, of all core procedures used in an establishment to prevent contamination or adulteration before and during production, the identification of the person(s) responsible for the monitoring and control of the GMP, and records of the actions undertaken to deal with deviations.

58.3.8 Management Commitment

The importance of management commitment to maintain and, where necessary, to improve hygiene standards can never be overemphasized. It is crucial that every company, irrespective of size, should have a coherent policy for achieving the required hygiene standards and directing the appropriate allocation of resources (human, technical, and financial) to achieve this.

58.4 THE HACCP SYSTEM

58.4.1 General Considerations

The HACCP system is a systematic approach to hazard identification, assessment, and control.[24,65] It is primarily intended to be used by food producers to provide them with the ability to meet their responsibility to produce safe and wholesome foodstuffs. Originally it was developed in the United States (US) to provide astronauts with microbiologically safe foods for use on space missions.[3] Since it was first presented at a food safety conference in 1971, its use has been endorsed by governmental and trade bodies throughout the world.

HACCP can be used either prospectively or retrospectively. It is prospective when used during the design of a new product and development of its manufacturing process. It is retrospective when used for validating the safety of an existing process and product. In both cases, it is a dynamic, proactive, and iterative activity aimed at catalyzing or accelerating improvements in product characteristics, manufacturing procedures, and enhancing their control. The expected benefits from its use are improved productivity and reduced consumer risks.

Each specific HACCP study must define the scope of application and take account of any operations before or after the specific operation under study.[9] Visible support and commitment from senior personnel responsible for the food operation under study are essential for the implementation of an effective HACCP plan.[48,49]

There may be some confusion between the process of risk assessment as used by a health authority as part of risk analysis[63] and the use of risk assessment as part of the hazard analysis process carried out within HACCP. It is important to appreciate that the two types of assessment are quite different both in scope and objectives. Thus the process of risk assessment as used by a health authority is a government-led procedure aimed at assessing the health risks to a population or subpopulation, arising, for instance, from the contamination of a foodstuff with a foodborne pathogen. Risk assessment as applied within the HACCP process, on the other hand, is an industry-led procedure, and concerns assessing the probability of occurrence of particular microbiological hazards associated with the production of a foodstuff by a specific process in a particular food establishment. This confusion between the different uses of risk assessment can be avoided by use of "hazard assessment" for the risk-based part of the hazard analysis stage of HACCP.

There are important links between government-led risk assessment and HACCP as shown in Figure 58–2.

First, government-led risk assessment may provide quantitative information on the concentration of a microorganism or a toxin that is potentially of health concern in a foodstuff, and thus provide those concerned with the development of an HACCP plan information on a concentration that must not be exceeded in a marketed food. Second, the outcome of the risk management process (an activity consequential on risk assessment and where options for control of an unacceptable risk are considered) may lead to the proposal of a specific food safety objective (ie, a statement of the maximum level, frequency, or concentration of a hazard in a food that is tolerable in relation to an appropriate level of protection), which may require industry to apply a control procedure during the production of a foodstuff such as a heat process giving a defined reduction in the num-

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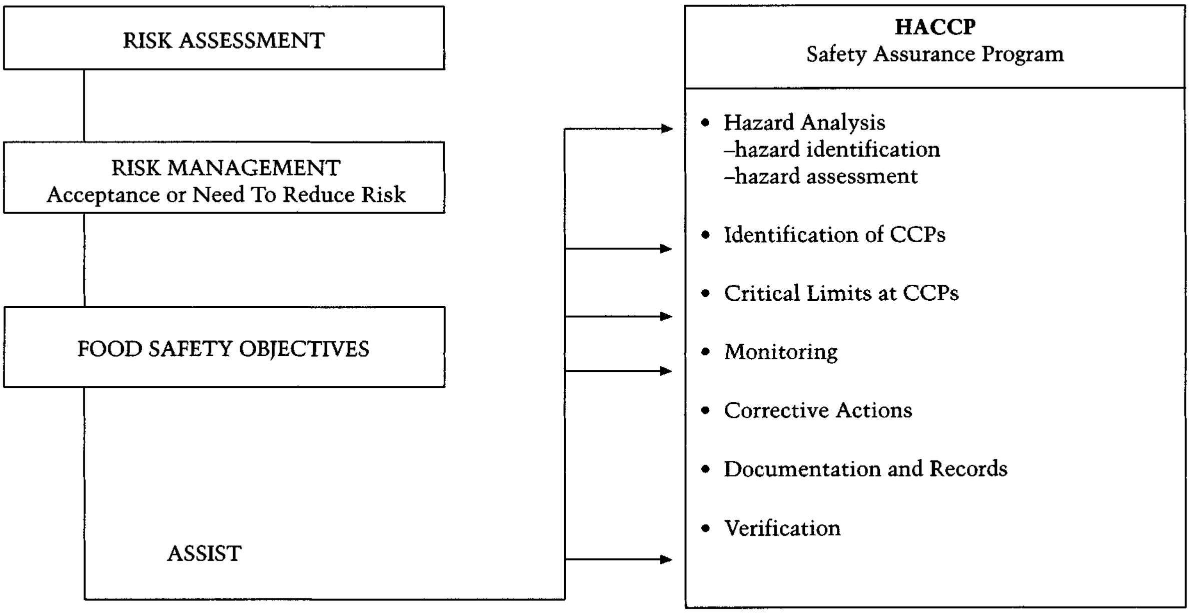


Figure 58–2 HACCP and Risk Assessment

ber of a pathogen. Third, it may provide information on the likely source(s) of microorganisms/toxins in, for instance, raw materials or ingredients.[6,51] Finally, it may provide guidance on the identification of potential critical control points (CCPs) and their control requirements (including verification).

Recognizing that much has been written on the value and application of HACCP (eg, ICMSF*,[24] NACMCF*,[46,47] CFDRA*,[9] IAMFES*,[23] Bryan,[5] Pierson and Corlett,[54] Mortimore and Wallace,[44] ILSI[31]) and there is an internationally agreed-on approach to establishing and applying HACCP, proposed by the Codex Alimentarius Commission, the latter will be used as the main basis for the following sections on HACCP.

58.4.2 Use of the Codex Alimentarius Approach to HACCP[12]

The main features of this approach are that it sets a framework for the use of HACCP that is applicable throughout the food chain, it provides an internationally accepted glossary of terms (see Exhibit 58–2), and it enunciates seven key principles or steps in setting up an HACCP plan.

These principles are:

1. Conduct a hazard analysis
2. Determine the CCPs
3. Establish critical limit(s)
4. Establish a system to monitor control of the CCP
5. Establish the corrective actions to be taken when monitoring indicates that a particular CCP is not under control
6. Establish procedures for verification to confirm that the HACCP system is working effectively
7. Establish documentation concerning all procedures and records appropriate to these principles and their application.

It also provides guidance for the application of these principles based on a sequence of 12 activities. These are:

1. Assemble an HACCP team
2. Describe the product
3. Identify the intended use
4. Construct a flow diagram
5. Carry out onsite confirmation of the flow diagram
6. List all potential hazards associated with each

**ICMSF*, International Commission for Microbiological Specifications for Foods; *NACMCF*, National Advisory Committee for Microbiological Criteria for Foods; *CFDRA*, Campden Food and Drink Research Association; *IAMFES*, International Association of Milk, Food, and Environmental Sanitarians; *ILSI*, International Life Science Institute.

Exhibit 58–2 Glossary of Terms Used in HACCP

Control (verb): To take all necessary actions to ensure and maintain compliance with criteria established in the HACCP plan.
Control (noun): The state wherein correct procedures are being followed and criteria are being met.
Control measure: Any action and activity that can be used to prevent or eliminate a food safety hazard or reduce it to an acceptable level.
Corrective action: Any action to be taken when results of monitoring at a CCP indicate a loss of control.
Critical control point (CCP): A step at which control can be applied and is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level.
Critical limit: A criterion that separates acceptability from unacceptability.
Deviation: Failure to meet a critical limit.
Flow diagram: A systematic representation of the sequence of steps or operations used in the production or manufacture of a particular food item.
HACCP: A system that identifies, evaluates, and controls hazards that are significant for food safety.
HACCP plan: A document prepared in accordance with the principles of HACCP to ensure control of hazards that are significant for food safety in the segment of the food chain under consideration.
Hazard: A biological, chemical, or physical agent in, or condition of, food with the potential to cause an adverse health effect.
Hazard analysis: The process of collecting and evaluating information on hazards, and conditions leading to their presence, to decide which are significant to food safety and therefore should be addressed in the HACCP plan.
Monitor: The act of conducting a planned sequence of observations or measurements of control parameters to assess whether a CCP is under control.
Step: A point, procedure, operation, or stage in the food chain, including raw materials, from primary production to final consumption.
Validation: Obtaining evidence that the elements of the HACCP plan are effective.
Verification: The application of methods, procedures, tests, and other evaluations, in addition to monitoring, to determine compliance with the HACCP plan.

Source: Reprinted with permission from *Food Hygiene—Basic Texts*, pp. 34–35, © 1997, U.N. Food and Agriculture Organization.

step, conduct a hazard analysis, and consider any measures to control identified hazards
7. Determine CCPs
8. Establish critical limits for each CCP
9. Establish a monitoring system for each CCP
10. Establish corrective actions
11. Establish verification procedures
12. Establish recordkeeping and documentation.

This approach is very similar to that recommended by the NACMCF[46] and has served as a common format for most other contemporary descriptions of HACCP.[9,31,64]

The following sections will consider in turn each of the 12 activities recommended by Codex for the application of HACCP.

Assemble HACCP Team

The design of an HACCP system for a particular product requires a multidisciplinary team approach. The scope of the study influences the composition of the team that should include the range of experience and in-house, or where necessary external, expertise to properly carry out the activities identified. According to the particular study, the team might include a production specialist responsible for, or directly involved in, the process under study; an engineer (with knowledge of the technical performance of any processing equipment and aspects of design that may affect product hygiene); a quality assurance/quality control specialist; and other relevant experts (eg, a food microbiologist with specific knowledge of the microbiological implications of the particular processing operations). A team leader should be nominated to guide the discussions and a secretary to record the decisions. For small organizations, it may be appropriate for one person to fulfill more than one role provided that the team is capable of obtaining and using relevant information to identify the hazards potentially associated with a particular production operation, to assess their significance, and identify options for their control.[9,23,44,46,47,63] For some companies, this may prove difficult or impossible, and, in the absence of suitable in-house expertise, a third-party organization may need to be used to provide the required level of expertise to develop a valid HACCP plan.

Describe Product

Such description should include the raw materials and the final product. For each raw material used, collect data on the type, source, purchasing specifications, and its percentage use in the final product (see below). Also any procedures or treatment(s) applied before receipt, conditions of storage, and additional data such as pH or a_w of the raw materials should be recorded. The final product, or intermediate products as necessary, should be defined in terms of composition, structure, processing conditions (eg, heat treatment), intrinsic factors affecting stability (eg, pH, a_w, preservatives), packaging, storage and distribution conditions (temperature in particular), shelf life expectations, package labelling, and instructions for use. A useful system for collecting and recording such information is illustrated in Exhibit 58–3.

In this system, for each raw material used, data are collected on its type, its percentage in the final product, conditions of storage, and additional data relevant to its microbiological stability such as pH and a_w. For each process operation, data are collected on the type of operation, the equipment used, the product and environment time/temperature, and additional information on the equipment such as materials of construction. The information in the boxes can be linked together to indicate product flow(s) and to form a detailed outline of the process.

Identify Intended Use

The intended use should be based on the expected uses of the product by the end user or consumer. In addition, it is necessary to take into account the possibility of exposure of the final product to temperature abuse by the purchaser and the likelihood that it will be consumed by vulnerable groups of people including infants and children, pregnant women, the elderly, or people with impaired health.

Construct Flow Diagram

Description of the process requires the preparation of a process flow diagram. The diagram should cover the whole operation from the selection of raw materials through the processing, packaging, final storage, and distribution stages and show the breakdown of the process in each constituent step. The flow diagram should be accompanied by specific technical information. These may include factory, line, and equipment lay-out; flow conditions of products, including delays during or between steps, recycle or rework loops, and transfers between steps; routes of mobile materials, equipment, and utensils; air flow and water flow; personnel routes; and segregation of any low- or high-risk areas.

In addition, for each process operation, data should be collected on the type of operation and its technological function (eg, effect on microbial flora and numbers), equipment characteristics (eg, size, capacity, existence of void space), operation parameters, product and environment time and temperature conditions, cleaning and disinfection procedures, and environmental and personnel hygiene.

A conventional flow diagram containing the type of information that should be recorded is illustrated in Figure 58–3. This type of diagram can be used to record the information described above or the flow diagram can be built up from the series of boxes illustrated in Exhibit 58–3.

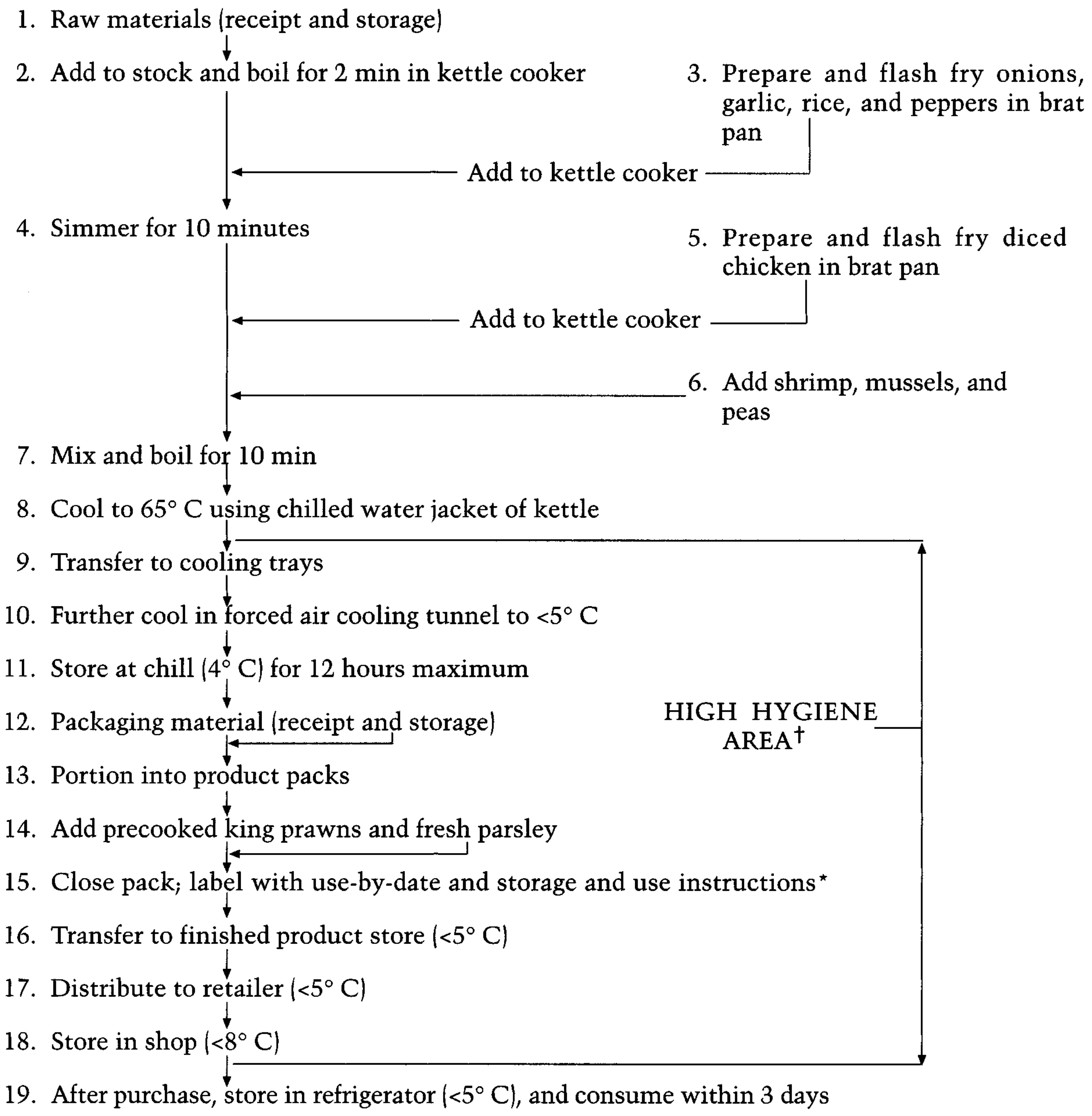
Carry Out Onsite Verification of Flow Diagram

The flow diagram should accurately reflect the actual processing operation. For an existing product/

Exhibit 58–3 Example of Data Collection and Recording System

Each Raw Material	*Each Process Stage*
Name and Source—% addition of raw material to product	Name of equipment—process size, capacity stage
Storage Conditions—pH and a_w (time/temperature)	Time/Temperature of process material(s) and materials of construction of equipment (void spaces?)

Source: Reprinted with permission from T. Mayes and D.C. Kilsby, The Use of HAZOP Hazard Analysis to Identify Control Points for the Microbiological Safety of Foods, *Food Quality and Preference*, Vol. 1, pp. 53–57, © 1989, Elsevier Applied Science.


1. Raw materials (receipt and storage)
2. Add to stock and boil for 2 min in kettle cooker
3. Prepare and flash fry onions, garlic, rice, and peppers in brat pan
Add to kettle cooker
4. Simmer for 10 minutes
5. Prepare and flash fry diced chicken in brat pan
Add to kettle cooker
6. Add shrimp, mussels, and peas
7. Mix and boil for 10 min
8. Cool to 65° C using chilled water jacket of kettle
9. Transfer to cooling trays
10. Further cool in forced air cooling tunnel to <5° C
11. Store at chill (4° C) for 12 hours maximum
12. Packaging material (receipt and storage)
13. Portion into product packs
14. Add precooked king prawns and fresh parsley
15. Close pack; label with use-by-date and storage and use instructions*
16. Transfer to finished product store (<5° C)
17. Distribute to retailer (<5° C)
18. Store in shop (<8° C)
19. After purchase, store in refrigerator (<5° C), and consume within 3 days
HIGH HYGIENE AREA†

*use-by-date is 12 days from manufacture
†control air temperature and humidity, hygiene of equipment, and hygienic practices of personnel

Figure 58–3 Flow Diagram of Model Process for the Production of Ready-To-Eat Chill-Stored Paella. *Source:* Reprinted from S. Mortimore and C. Wallace, *HACCP A Practical Approach*, © 1994, Aspen Publishers, Inc.

process, the collection of information must be carried out under production conditions and require close observation of all stages, including any night shift or weekend running. A final onsite confirmation during hours of operation will provide an appropriate verification of the data collected.

Where a new product development is under evaluation, data representing the product definition and the most likely processing options are provided by the departments involved in the development.[24] These will require confirmation and may possibly need some revision when the product is finally put in to production. Carrying out a preliminary HACCP at an early stage of product development will identify the main potential CCPs and highlight control needs.

Perform a Hazard Analysis

Hazard analysis is the process of collecting and evaluating information on hazards associated with a particular food that are potentially of health concern (together with any specific conditions potentially leading to their presence at health concern levels), to decide which are significant for food safety and, therefore, should be addressed in the HACCP plan. Hazard analysis, therefore, includes two stages: hazard identification and hazard assessment.

When developing a safety assurance plan, all potential biological, chemical, or physical hazards associated with any step, point, or procedure in the process need to be identified by mean of a stepwise investigation. The current discussion, however, will focus only on microbiological hazards and include spoilage as well as health concerns.

Hazard Identification. Microbiological hazard identification encompasses a listing of all types of microbiological hazards relevant to the product under consideration and the specification of the conditions (eg, failure to control a particular raw material or processing step) leading to the presence of microbiological contaminant(s) at hazardous levels (ie, levels that are likely to cause illness or spoilage). Such conditions may occur at any stage in the food operation under study resulting in the growth or survival of an organism or toxin in a foodstuff or the contamination or recontamination of a raw material, intermediate, or final product.[31]

Listing of Types of Microorganisms and Microbial Toxins. Both food poisoning and spoilage microorganisms (and their metabolic products) are of concern, although only pathogens and toxins would be considered in a specific safety assurance plan. An appreciation of those hazards that are relevant to the product and process under consideration is the natural starting point of a hazard analysis. It is also the most difficult part of the analysis because unless this activity is done thoroughly, using knowledgeable people with appropriate understanding of the food operation under study, the subsequent decisions on product safety requirements may well be flawed. Thus, this step requires microbiological knowledge of the physiology of microorganisms of potential concern in the foodstuff, and specifically the influence of intrinsic, extrinsic, and implicit factors relevant to the food; processing; storage and use conditions; their destruction, survival, or growth; or their ability to form toxins and the resistant characteristics of these.[26,45] It also requires knowledge of the microbial ecology of raw materials used, the factory environment, and products (information that may only be obtained by a microbiological investigation carried out on the actual plant). Also needed is technical information on the major processes, any epidemiological information indicating whether a given product or a similar product has been implicated in foodborne disease and the type(s) of microorganisms involved, and commercial or other information (such as results of challenge testing or storage trials) on the microbiological stability and safety of the product.

All this information is used to determine which microorganisms the study needs to consider. Starting with a list of known pathogens (and spoilage microorganisms where relevant), the exercise consists of selecting relevant microorganisms and toxins likely to be of concern. It may be possible to group together similar organisms of concern and use one organism that is representative of that group on the grounds of similar physiology and survival characteristics. The chosen organism should be a likely contaminant and be most resistant to the conditions used in production and, where appropriate, able to grow in the final product. Other important points to consider in selection of microorganisms of concern are:

- the type (and number) of microorganisms expected to be present in raw materials
- the type (and number) of microorganisms likely to be introduced during handling or processing
- the effect of processing or handling, including consumer practices such as preparation for consumption, on their growth, survival, or death
- the target group(s) of consumers.

It is most important that all relevant potential hazards be identified without regard to their probability of occurrence or their severity. For instance, although botulism is properly controlled by the thermal process used by industry for the manufacture of low-acid canned foods, when embarking on an HACCP study of such a process, a food producer should always list *Clostridium botulinum* as a potential hazard for such type of production. This will allow the HACCP team to decide, as the analysis proceeds, whether the conditions of production and the status of controls currently in use are satisfactory or need improvement. A similar reasoning applies also to new or emerging pathogens such as verocytotoxin-producing strains of *Escherichia coli*, which need to be included in HACCP studies of ecologically relevant products. Where microbiological specifications have been developed for a foodstuff (particularly if these have been based on a risk assessment), they may be used as an aid in identifying microorganisms of concern for that specific foodstuff and the level to which they should be controlled. However, hazard identification should not only be limited to those microorganisms covered by existing specifications but should include consideration of all relevant foodborne pathogens, toxins, and spoilage microorganisms.

The HACCP team should examine systematically the particular microorganisms or microbial toxins that may affect the safety or "keepability" of the product(s) under consideration. In this way, a list of likely microbial hazards, based on known information, is obtained.

Conditions Leading to the Presence of Microorganisms and/or Microbial Toxins. For each identified hazard, the analysis then proceeds with the identification of situations where the hazard(s) listed under the previous section could be introduced into the production process. The exercise is done stepwise for each raw material and ingredient and for each step in the operation, including packaging, storage, and distribution, using the process flow diagram as a guide. Thus each raw material or ingredient is evaluated for the likely presence of microorganism(s) and/or toxin(s) identified to be of concern.

Each processing or food handling step identified in the flow diagram is systematically and specifically considered in sequence as to its possible role in creating or controlling a microbiological hazard. This would include the effect of possible delays during and between processing steps, rework loops, and conditions of transfer from one step to the other on the growth of microorganisms of concern. Also, the potential at each step for contamination (or recontamination) or process failure leading to survival of hazards of concern is systematically evaluated.[42] As the analysis proceeds, the likely conditions and causes leading to the realization of these hazards are recorded and, as far as possible, the effect of these on microbial numbers quantified. During these activities, the HACCP team should consider not only what could happen under normal operating conditions, as recorded in the process data collected, but also under realistic process variation, malfunction, drift, or misoperation at any step in the production process. For each of the hazards considered, this leads to a listing of conditions under which a significant hazard could occur for all raw materials and processing steps throughout the food operation under study.

Several tools or techniques can be used to structure and quantify the process of hazard identification, including the use of standard questionnaires such as those proposed by bodies such as ICMSF,[24] IAMFES,[23] and NACMCF,[46] and quality improvement tools developed by quality assurance specialists. Among the latter, cause and effect analysis[39] has been found to be useful. This considers, during a brainstorming session, the relationship between different categories of possible causes and a given effect (eg, insufficient heat treatment and microbial survival). Five major categories of causes may be investigated in this exercise (ie, the environment, equipment, production methods [including process parameters and measurements], food materials, and personnel). Each category of major cause is analyzed and brainstormed to focus-in on more and more specific causes. Other structured, reliability analytical techniques include Fault Tree Analysis,[39] Failure Mode and Effect Analysis,[3] and HAZOP;[2] the latter has been found to be particularly valuable for structuring microbiological hazard analyses.[42]

Other useful data can be obtained by measurements of temperature, pH, and a_w of the product and testing of product and environment samples for microorganisms.[5,23] Also the use of challenge testing,[53] predictive microbiology, and related software (see Chapter 16) will provide further additional information on conditions likely to lead to a microbiological hazard.

Hazard Assessment. Hazard identification results in a list, identifying for each microbiological hazard the conditions leading to its occurrence. While clearly all such hazards and condition(s) under which they may occur should be appropriately controlled, to decide the degree to which resources should be applied to their control, it is necessary to decide which of the identified hazards will significantly affect the safety or wholesomeness of the final product. Such a hazard assessment should take into consideration two factors: the frequency or probability of occurrence of a condition(s) that could result in the hazard(s) occurring, and if these occur, their seriousness in terms of adverse health effects or a spoilage concern. Thus, the expected outcome of such an assessment is a ranking of the significant hazards, a listing of the condition(s) that could lead to their occurrence, and the likely frequency and seriousness of such an event if it occurred. This information is to be used when control setting, determining priorities for action, and specifying the stringency of control required.

The frequency of occurrence of a hazard relates specifically to how the conditions applied during a food operation lead to the presence of the hazard in the final product. For instance, the presence of salmonella in a dry milk powder is a general cause of concern for this type of product, and salmonella would be expected to occur in any listing of relevant hazards to be considered in an HACCP study of a dry milk plant. After identifying the conditions leading to the potential presence of Salmonella in the specific production operation under study, it is then necessary to assess the probability of occurrence of such conditions. Assessing such probability consists, basically, of answering two questions. First, does the condition leading to a significant hazard occur only very occasionally, or, conversely, is it an ordinary or usual situation? Second, as a result of the condition occurring, will the hazard concerned be present only occasionally in the final product, and how often, or will it be regularly present?

Such frequency estimation is ideally based on the number of times the condition leading to a hazard occurring has actually been observed to occur (ie, based on a documented history of processing successive food lots). In the absence of such information, an estimate or probability of occurrence should be made based on the best information available on the occurrence of the hazard and likelihood of the condition that could lead to its occurrence during expected production conditions. Such an assessment, if based on observed frequency, may be quantitative but is more often characterized by a qualitative scale such as high, medium, or low or definite, likely, or remote. Such an estimation should be based on technical information and expertise and will often require the use of brainstorming or some other investigative technique to obtain the best estimate.

Determining the severity or gravity of a hazard requires two considerations. The first is to consider, for each identified microbiological hazard, the consequences of the occurrence of each hazardous condition on the types and numbers of microorganisms or metabolites that could be present in the final product. For example, the number of pathogenic or spoilage microorganisms originating from a raw material that are likely to be present in a final product may depend on the formulation of the product, the effect of subsequent processing steps, and line or environment cross-contamination by raw materials. Also, the effect of process inadequacies or failures on type and numbers of microorganisms should be considered, as well as in- or post-process contamination, mishandling during storage, distribution, retail display, or consumer use. Predictive microbiology can be used to simulate the effect of these possible deviations on microbial growth or survival—provided the conditions arising from them can be measured or estimated; the use of such simulations is discussed in Chapter 65. All of this will provide information on hazard occurrence in the final product.

The second, and most important consideration, is an appreciation of the significance of the occurrence of a hazard. Will it result in damage to the consumer? What is the gravity of the disease arising? Alternatively, are the consequences trivial or insignificant in terms of foodborne disease or product spoilage? Where there is a need to meet a legal or customer-specified microbiological criterion, will this lead to nonconforming lots?

To reach a meaningful decision on the severity of a hazard, human and technical factors need to be taken into consideration (eg, the target consumer group). For instance, considering our example of salmonella in dry milk, salmonellosis is a severe disease for the population at large. However, the consequences of the presence of salmonella in dry milk would be of even greater concern, and hence severity, if the product is specifically intended to be used for feeding infants.

Severity of a hazard should be quantified as far as possible, taking account of the considerations listed above. However, very often severity can only be expressed qualitatively as high, when directly resulting in the potential of an unsafe or otherwise unacceptable product; major or moderate, when reducing the acceptability of a product for its intended purpose; and minor, when it is not likely to reduce usability.

The qualitative and, where possible, quantitative assessment of frequency of occurrence of a hazard and an estimate of its severity are finally used to rank, according to degree of concern, the process stages, practices, or procedures that potentially could lead to a hazardous situation(s) or condition(s).

Ranking usually is expressed by such terms as "critical," "major," or "minor" concern. Specific techniques, such as the use of failure mode, effect and criticality assay,[8,39] can be used to structure this activity.

Determine Critical Control Points

The process and conclusions of hazard analysis allow identification of places in the food operation where hazard-causing situations may occur, together with their degree of concern. The need now is to handle this information to decide on how to manage such situations.

Having first checked that GMP requirements are in place, the HACCP team then should consider what additional control measures (if any) are required. One decision that may arise is the possibility of totally eliminating a hazard or of reducing the impact of a hazardous situation or condition by introducing changes in the product or in the process. This may require changing the form or source of a raw material or ingredient; changing the formulation of the product, such as the introduction of new microbiological barriers; changing a step in the manufacturing process or its processing parameters; modifying the conditions of packaging or distribution; or modifying the instructions for preparation for consumption.

The control of the remaining hazardous conditions is based on the use of the concept of a CCP. This can be defined as a step at which control is essential to prevent or eliminate a food safety hazard (identified by the previously described process of hazard analysis) or to reduce it to an acceptable level.[12] The rationale behind the identification of a CCP is that if an operation is faulty and/or monitoring inefficient at this point, this will result in a major concern and a high probability that a severe, direct health hazard or a high level of spoilage will occur. On the contrary, if a CCP is properly controlled and monitored, it will provide a high degree of confidence that the identified hazard will be effectively controlled.

To be effective, CCPs have some basic characteristics.[59] First, they must be specific points where control is necessary to avoid the occurrence of one or more hazards. Second, operational means must be readily available for their control and at a reasonable cost. Such means can be directed toward eliminating a microorganism of concern or reducing its number to an acceptable level (eg, use of pasteurization to kill infectious disease-causing pathogens such as salmonella in milk and to increase the shelf life of milk) or preventing a hazardous condition from occurring or reducing its impact (eg, by acidification or refrigeration of a product to slow or prevent microbial growth). Finally, they must be capable of being readily and reliably monitored. For example, they could be monitored by visual means such as observation, by physical means such as measurement of pH or temperature, or by chemical means such as monitoring the level of chlorine in the water used for cooling canned food after a thermal processing.

CCPs are established with specific regard to each significant hazard identified. In many instances, they correspond directly to a step, practice, or procedure from which a hazardous condition may arise if control is lost. In some instances, direct control of a hazardous condition where it occurs is not possible, is difficult to achieve, or effective monitoring is not feasible. In such cases, the possibility of establishing CCPs further in the process should be considered. For instance, in the canning industry, avoidance of understerilization is achieved by appropriate control of temperature and time of the sterilizing process. After sterilization, it is necessary to ensure that recontamination (a hazardous condition) cannot occur. Recontamination can occur as a result of the presence of minute holes in the sterilized container and organisms being drawn into the container during cooling. Although the sealing process, applied to the container prior to sterilization, is a CCP, it is difficult to control this process such that no holes in the seams or seals of the containers occur; also monitoring techniques are not able to guarantee that all containers are free from holes. Therefore, CCPs must be established after sterilization to prevent contamination entering the container and contaminating the product. Control measures include chlorination of the water used for cooling the containers and the avoidance of manually handling the containers while they are hot.

Identification of CCPs requires a good understanding of the food operation, technical expertise, and expert judgment. To facilitate this task, it may be advantageous to use "decision trees" as guidance. Examples of decision trees aimed at identifying CCPs can be found in the literature.[12,31] One such decision tree, developed by a working group set under the auspices of the Codex Alimentarius Food Hygiene Committee, is illustrated in Figure 58–4.

In this decision tree, for each step in the process with identified hazard(s) (starting with the raw materials and proceeding stepwise throughout the process), each of the four questions listed in Figure 58–4 is considered and answered in sequence.

While there is no lower or upper limit to the number of CCPs that can be identified in a process, it is always wise to keep the number of CCPs to a minimum to keep the system "user friendly"; always remember that general hygiene requirements are con-

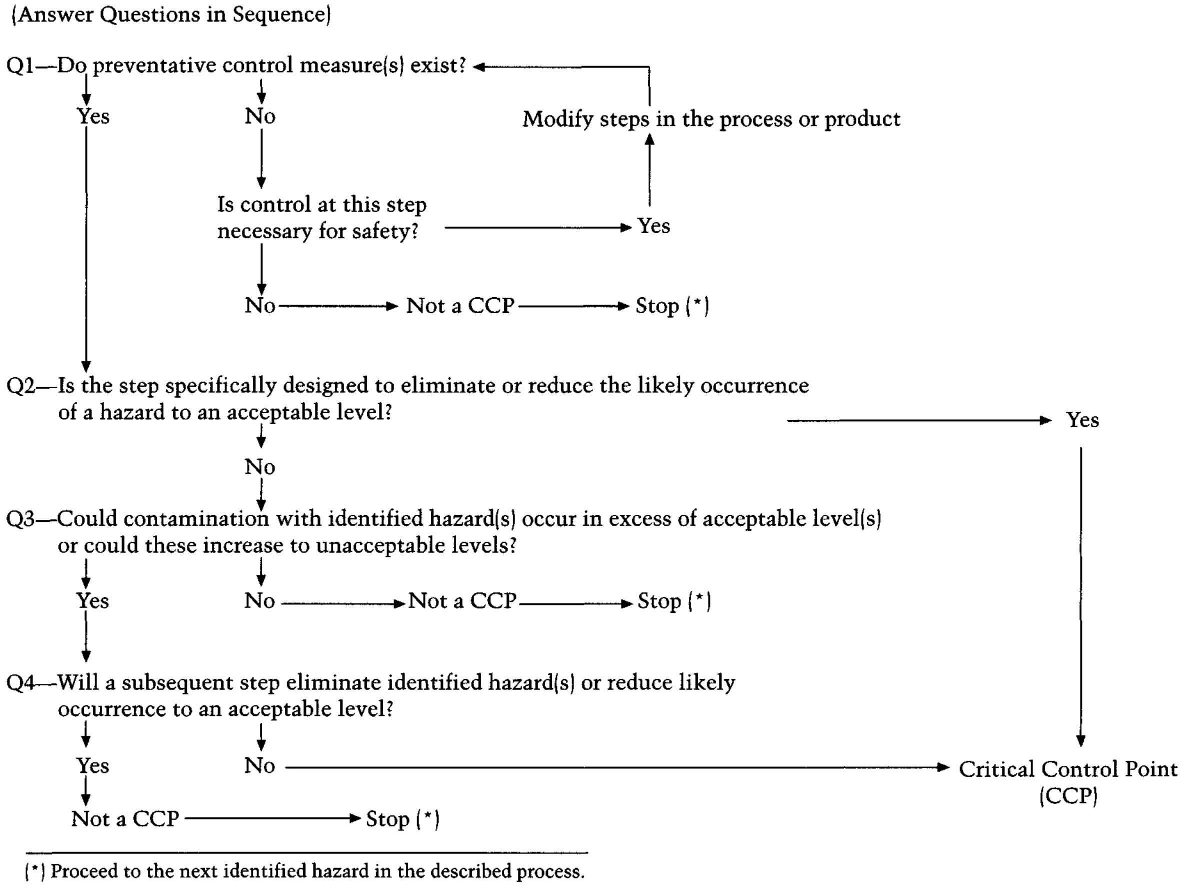

(Answer Questions in Sequence)
Q1—Do preventative control measure(s) exist?
Yes
No
Modify steps in the process or product
Is control at this step necessary for safety?
Yes
No
Not a CCP
Stop (*)
Q2—Is the step specifically designed to eliminate or reduce the likely occurrence of a hazard to an acceptable level?
Yes
No
Q3—Could contamination with identified hazard(s) occur in excess of acceptable level(s) or could these increase to unacceptable levels?
Yes
No
Not a CCP
Stop (*)
Q4—Will a subsequent step eliminate identified hazard(s) or reduce likely occurrence to an acceptable level?
Yes
No
Critical Control Point (CCP)
Not a CCP
Stop (*)
(*) Proceed to the next identified hazard in the described process.

Figure 58–4 Example of Decision Tree To Identify Critical Control Points. *Source:* Reprinted with permission from *Food Hygiene—Basic Texts*, p. 44, © 1997, U.N. Food and Agriculture Organization.

trolled as part of GMP. Check that CCPs initially identified are not redundant (ie, although control of a particular step appears necessary to prevent a hazardous condition arising such as the potential for an acceptable growth or survival of a foodborne pathogen, and is therefore a CCP, a later step in the process may also provide effective control, and is also a CCP, making the first CCP redundant). This duplication of CCPs should be avoided by following the logic sequence of questions in Figure 58–4. Further CCPs may be eliminated by introducing changes in the product or process; in addition to reducing the number of CCPs, this can often also make the product safer.

If an analysis suggests that a potentially critical hazardous condition cannot be controlled at a certain step in the process or production operation, and no later CCP will give effective control, this should trigger modification of the step (eg, equipment or procedure) or the reformulation of the product to introduce a CCP.

It cannot be overemphasized that CCPs are highly specific to a given product and the conditions used for its production; they must not be applied to another food operation without first checking, by way of a hazard analysis, that they will apply. Any change in the product/process also should trigger a new hazard analysis and, if necessary, the revision of the list of CCPs. Due to such specificity, CCPs listed in so-called generic HACCP models (ie, HACCP models developed for the manufacture of a particular commodity or food product and not related to a particular plant or specific manufacturing operation) may be totally misleading and reduce the flexibility inherent in the proper use of HACCP. Their use should, therefore, be generally discouraged and avoided except for their possible use for illustrative purposes in general hygiene codes, in which cases their purpose must be clearly stated and the reader warned not to apply them to a particular operation without first performing a hazard analysis.

For each CCP, the control measures that can be applied for each hazard have to be identified and documented. These are intimately associated with the concept of a CCP. They refer to the techniques and activities that give the CCP its function in preventing or eliminating a hazard or reducing it to acceptable level. The control measures at each CCP should be selected in terms of effectiveness and utility. This principle can be illustrated with reference to the control measures applied to ensure the microbiological acceptability of pasteurized ham. Here, design and maintenance of equipment and operational control (eg, time and temperature of processing and hygienic handling of cans postprocessing) are more effective (high success and low cost) than sampling and testing for indicators of underprocessing or postprocessing recontamination (low success and high cost).[2]

Also effective control of the raw material supply, where a raw material is identified as a CCP, should include supplier approval, based on the supplier's safety assurance plan (HACCP-based) and an established quality assurance program. This will give much greater assurance that a delivery of a raw material is acceptable for use rather than relying on testing on receipt; for information on the confidence of decisions based on microbiological testing and on the application of microbiological criteria, see Chapters 60 and 65 respectively.

Establish Critical Limits for Each CCP

A critical limit is defined as a criterion that separates acceptability from unacceptability.[9] The purpose of a critical limit is to provide a basis for the acceptability of control applied at a CCP. Such definition and purpose have three crucial implications for the management of a CCP.

First, a critical limit marks the absolute tolerance of a control measure applied to a CCP and should never be exceeded. When a critical limit is exceeded, the CCP is out of control and the product is likely to nonconform (eg, potentially a safety concern or having nonconformance with pre-established specifications). When day-to-day values are used (eg, target values or tolerances), they should always be established within the critical limits and be so designed that they ensure that the critical limits will not be transgressed, while taking into account normal process variations and measurement errors. For instance, a pasteurizer in a milk plant could be operated so that the milk is pasteurized at a target temperature of 73° C and held at this temperature for 15 seconds. The critical limit for this process is to heat the milk to a temperature of 71.5° C for 15 seconds, which is the generally accepted minimum limit for high temperature short-time pasteurized milk. However, the plant is operated at the higher temperature so that no milk will be pasteurized at a temperature below the critical limit.

Second, a critical limit should be quickly and easily observed or measured and provide a yes/no response. Thus where control of a microbiological concern is the intent, it is preferable to use a physical or chemical parameter as a critical limit rather than a microbial concentration because the latter often is difficult to measure accurately (see Chapter 60) and the results of an analysis slow to obtain. Parameters that can provide good information on the state of control of microbial hazard include physical parameters such as time, temperature, humidity, moisture level, a_w, and pH, and chemical parameters such as titratable acidity, salt concentration, available chlorine, viscosity, and concentration of preservatives. They also may include the use of behavioral (eg, compliance with a specific food handling procedure) or sensory parameters (eg, visual appearance, texture, aroma). Although critical limits based on direct microbial numbers are not generally useful for reasons stated above, where very rapid measurements can be made (eg, ATP bioluminescence to monitor cleaning effectiveness or impedance measurement to monitor total levels of microorganisms in raw materials), the related parameter can be used to set critical limits.

Third, a critical limit, in order to be significant, must be coherent with the microbiological requirements (including microbiological specifications) defined for the final product. Only by taking into consideration such requirements can appropriate critical limits be identified at a single processing step, a sum of steps, or even for the whole process. Evidence of such relationship should always be established and provided. To that aim, the microbiological requirements for the product produced by the operation to which the HACCP plan is under development should be known or agreed to in advance by the HACCP team. Such microbiological requirements may be expressed as a performance criterion (eg, a specified reduction in microbial numbers such as the 12D concept applied for the destruction of *C. botulinum* in low-acid canned foods), a process criterion (eg, the time and temperature used to reduce *C. botulinum* by 12D), or a microbiological criterion (eg, a limit for a concentration of a given microorganism or toxin in a product); the relationship between these different types of criteria is discussed in Chapter 65.

Critical limits can be derived from many sources: a risk assessment; research; scientific and technical literature; computer simulation; in-house experiments; supplier's records and data; expert advice and consultancy; guidelines from international organizations; and regulations. For existing products, limits already in place should be assessed and validated prior to their use in the detailed HACCP plan. Where necessary, a change in parameters and/or values may be introduced.

Establish a Monitoring System for Each CCP

Monitoring is the scheduled measurement or observation of a CCP relative to its critical limit(s).[12] The rationale for monitoring is to provide evidence that critical steps or procedures (ie, CCPs) are kept under control and that, should an out-of-control situation occur, it can be readily identified so that corrective action can be taken rapidly. It involves the scheduled observation or measurement of significant components or parameters at CCPs, to assess whether they are under control.

Monitoring should aim to identify an out-of-control situation at a CCP before start-up or during production. Ideally, systems should be in place that monitor control at CCPs continuously, online and in real-time, thus providing immediate and automatic adjustment as necessary. Most frequently, however, only discontinuous and/or offline monitoring systems can be used. For such, it is necessary to ensure that the frequency of observations and/or sampling should be statistically valid. Whenever practical, statistical process control techniques should be used to enable objective decisions for corrective actions to be reached; see Chapter 60.

Monitoring techniques have been reviewed extensively.[24] As noted previously, visual observations, sensory evaluation, and physical or chemical measurements are best suited; microbiological methods (except the very rapid ones, eg, ATP bioluminescence) are not appropriate for monitoring. Microbiological methods are nevertheless invaluable for investigating a process and may be useful for verification purposes; see Chapter 65.

The monitoring procedures should be fully documented with indication of the CCPs to which they apply, the characteristic(s) to be monitored, the frequency of monitoring, the method(s) to be applied, and the person responsible for approval of the records and for decisions on corrective actions. Full records must be kept of all monitoring data and the person responsible for circulation of information and records should be specified.

Establish Corrective Actions

Corrective actions are the actions to be taken when the results of monitoring indicate that the operation at a CCP fails to meet the critical limit (ie, a situation where control is lost leading to a hazardous condition). Such corrective actions need to be identified in advance and laid down in a corrective action plan.

The primary concern if a CCP is outside its critical limit is that the manufactured product does not, or may not, meet the specified requirements for safety. Hence, the corrective action plan should always document disposal of the suspect product—including identification, segregation, review, and appropriate disposition. This supposes some system of traceability and, in the case of products that have immediate delivery, an emergency or recall plan. The ISO 9004 standard[37] provides appropriate information and guidance on all such actions.

Another concern, of equal importance, is to prevent recurrence of the deviation that led to the critical limit being exceeded. This implies immediate action to adjust the processing or other conditions to bring the CCP back within its critical limit(s). It also requires a proper identification of all factors affecting the capability of the process to at all times meet the critical limits. Ensuing decisions are essentially based on the severity of the potential deviation that could occur. In a very high-risk situation (very high impact on food safety), it may be deemed necessary to stop the process until the problem is fully corrected. Otherwise, after adjustment of the process, specific additional monitoring or routine checks should be made to confirm that adjustment is effective in maintaining control. Should deviations occur with high frequency or control prove difficult to maintain, this should trigger, in addition to emergency corrective actions, verification and review of the HACCP plan. For effective prevention of problems caused by process drift, corrective actions to adjust the process before the drift leads to transgression of critical limits should always be carried out. Corrective actions should be fully documented and records kept on occurrence and actions taken.

Establish Verification Procedures

Once implemented, the HACCP plan and the resulting HACCP system should be evaluated on a regular basis. Within the HACCP methodology, this specific process is called "verification." Verification should be carried out primarily by the operator to confirm that the various elements of the HACCP plan are in place and the HACCP system derived from the plan is fully functional and provides the degree of assurance specified in the plan.

In contractual or regulatory situations, verification of the HACCP-based safety assurance plan also can be carried out by the customer or by a public authority to determine the ability of the food producer to meet specified requirements. The current discussion will focus on verification as used by a company for internal purposes. Several objectives may be involved in the process of verification. These include checking a plan prior to implementation, assessing whether a plan is properly implemented (ie, the HACCP system conforms with the HACCP plan; this preimplementation check may be called validation), determining whether a plan and system are functioning effectively, or reviewing the HACCP plan and system to decide on appropriate modification(s).

The frequency and extent of verification will depend on the evidence that leads the operator to conclude that the system is functioning properly. Thus, on implementation of a plan where there will be no evidence of proper functioning of a system, verification should always be carried out. As documented evidence of satisfactory functioning of the system is obtained, the frequency of verification can be reduced. Review of an HACCP plan should follow:

- frequent occurrence of process deviations
- receipt of information from the market indicating a health (or spoilage) problem
- when epidemiological or scientific information identifies new hazard(s) of concern
- when commercial information indicates changes in distribution systems or consumer's use or when anticipating such change
- prior to (rather than after) introducing changes in product/process conditions (eg, new raw material/product to be used, formulation to be changed, new processing technology/equipment to be used).

The basic technique for verification is to audit the HACCP plan and system or part of it. The procedures encompass all or part of the following.

Pre-audit Documentation Review. This would include review of documentation on GMPs (eg, hygiene measures in place, cleaning programs, personnel hygiene and training), HACCP documentation, and records.

Onsite Audit. This would include confirmation of product description, facility layout, and flow diagram; confirmation of hazards as identified; assessment/confirmation of CCPs; review of control options and critical limits and of their effectiveness; and confirmation of monitoring activities and of their effectiveness; review of adequacy and effectiveness of corrective actions; confirmation of the existing verification procedures.

Additional Verification Procedures. This could include, as necessary, collection and microbiological analysis of samples from CCPs or other appropriate points and storage tests on products (for instance, from a recently installed operation) or increased end-product testing. When the latter is considered, special attention should be given to the practicability of the microbiological methods involved and, more importantly, whether the microbiological quality level can be accurately assessed by a feasible sampling plan. Due to the statistical limitations of end-product sampling and testing,[20,24,25] once an HACCP plan has been properly designed, implemented, and appropriately verified, routine microbiological testing of end-products will serve little purpose and is therefore not recommended.

Audit Follow-Up. This would include the use of specific procedures to verify that the recommended changes have been introduced into the HACCP plan and satisfactorily implemented. The general questions that should be asked by an auditor, when verifying a HACCP system, are listed in Exhibit 58–4.

Establish Record Keeping and Documentation

Adequate documentation is essential to provide evidence that all issues relevant to the scope of the HACCP plan have been addressed, and controls properly designed, implemented, monitored, and verified. The documentation system involves three elements.

Documentation of the HACCP Plan. Documentation includes scope of plan; composition of the team involved and assigned responsibilities; description of raw materials, intermediate and final products, and their specifications; facility layout and flow diagram of the process; hazards identified (and reasons); CCPs identified; management of each CCP, including control measures, critical limits, monitoring activities, and corrective action plan(s); and verifica-

Exhibit 58–4 General Questionnaire for Use in HACCP Verification

Name of Plant: ____________________ **Date of Review:** __________________ **Name(s) of Reviewer:** _____________

Information To Be Reviewed: Much of the following information can be obtained prior to a review and verified on the plant visit by reviewing records and talking to those responsible for the HACCP plan(s), the plant operatives, and the plant management.

- Who is on the HACCP team? Who is the HACCP team leader?
- What are qualifications/experience of the team members?
- Has a member of the team attended an HACCP training course?
- Is there a documented HACCP plan for each process?
- Is there a flow diagram for each process?
- Is there a plant layout available? If so, do the product, process, and people flows within the plant minimize the possibility of microbial cross-contamination?
- Select an HACCP plan and review the process flow. Does it agree with the HACCP plan?
- Who were/was responsible for identifying the hazards and the CCPs in the particular HACCP plan(s) under review? Are the reasons for the choice of these documented? Are they soundly based? Have critical limits been established for each CCP? Who established the limits? What rationale was used for establishing these limits and their associated monitoring procedures? Are these soundly based?
- Who monitors a CCP? Is monitoring done according to the HACCP plan? Are the results properly recorded? Who verifies that the CCPs are monitored correctly? Do the operators know the critical limits and what to do to prevent a deviation from occurring (or is this done automatically)?
- What happens when a deviation occurs? Is a plan (documenting corrective actions) in place to address deviations? How is management notified of a deviation? Are all corrective actions undertaken when a deviation occurs properly recorded in a deviation log book or as a similar centrally maintained record? Who approves a change in a CCP, a critical limit, a monitoring procedure, or a corrective action? Are all changes approved, documented, and properly communicated to operatives, supervisors, and managers responsible for their implementation?
- Where are HACCP plan records maintained? Are they available for review by appropriately authorized personnel?
- Are all records pertaining to the control of CCPs available? Where and how long are they kept? Who is responsible for maintaining these records?
- Is the effectiveness of HACCP plan(s) verified by additional tests? Who is responsible for deciding what additional tests are appropriate and who reviews the results of such tests?
- Does the plant manager and his/her staff understand the HACCP concept and provide proper support for its adoption and maintenance? Who is responsible for HACCP training within the Plant? How effective is this training?
- On the basis of the review, have the HACCP plan(s) been properly established and implemented? Are there any recommendations for correction or improvement?

Source: Adapted with permission from R.B. Tompkin, HACCP in the Meat and Poultry Industry, *Food Control*, Vol. 5, pp. 153–161, © 1994, Elsevier Applied Science.

tion procedures. An example of a completed HACCP worksheet for the paella process illustrated in Figure 58–3 is shown in Exhibit 58–5.

Documentation Necessary for the Implementation of the HACCP Plan. This includes procedures and work instructions for operational activities, monitoring activities, corrective actions to be carried out at each CCP, and procedures for verification.

Records Obtained During the Operation of the HACCP System (Operation, Monitoring, Corrective Actions, Verification). The documentation system should be appropriately managed. The ISO 9004 Standard[37] provides appropriate guidelines. For the system to be effectively managed, documentation, as the system itself, needs be only as comprehensive as necessary and commensurate to the risk involved.

58.5 APPLICATION OF QUALITY SYSTEMS

A quality system is the organizational structure for responsibilities, procedures, processes, and resources needed to implement quality management.[33–37] It is designed primarily to meet the internal needs of a company for an organizational framework to coordinate all activities concerned with the implementation and management of quality. See Figure 58–5 for an outline of the main elements and functions of a quality system.

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Exhibit 58–5 Example of a Completed HACCP Worksheet

1. **Describe Product**—Chill-Stored Paella

2. **Diagram Process Flow**—see Figure 58–3

3. **List**

*Process Step**	*Hazard*	*Control Measure*	*CCP(s)*	*Critical Limits*	*Monitoring Procedures*	*Corrective Action(s)*	*Records*
1-Storage of raw materials	Presence of IVP‡; *B. cereus*	Supplier assurance correct storage	No See step 14				
7-Mix, boil 10 minutes	Presence of IVP; *B. cereus*	Time/temperature	Yes	98° C +/– 2° C 12 min +/– 2 min	Chart recorder	Isolate any undercooked lot	Chart
8-Cool to 65° C	Recontamination	Temperature	Yes	67° C +/– 2° C	Chart recorder or manual check each batch	Reheat if temperature below 65° C	Chart or manual record
11-Store at chill 4° C/ 12 h max	Recontamination and growth	Hygienic design and operation	Yes	Follow GMP for high hygiene area	Supervision of staff procedures	Reinforce hygiene Review procedures	Record actions
		Time/temperature		4° C +/– 1° C 12 h +/–2 h	Production lot records	Block product Inform QA	Record actions
14-Addition of king prawns	Presence of IVP	Supplier assur. microbiol. spec.	Yes	Within microbiological specifications	Intake testing according to supplier perform	Reject out of specification material; audit supplier	Record actions and findings
16–18-Store at 5° C during distribution and sale	Growth of contaminants	Time/temperature	Yes	Product temperature 5° C +/– 1° C max 12 days	Storage temps and date/lot code	Remove from sale if exceeds temp or outside date code	Record actions

*See Figure 58–3
†Steps 2, 6, 9, 10, 12, 13, and 15 are not CCPs
‡Infectious vegetative pathogens

Source: Reprinted from S. Mortimore and C. Wallace, *HACCP A Practical Approach*, © 1994, Aspen Publishers, Inc.

Managers are responsible for:

- defining and formally expressing a company's quality policy (ie, the overall intentions and direction of a company with regard to quality, including safety)
- defining and documenting lines of responsibility and authority
- establishing the organizational structure
- allocating appropriate and sufficient resources
- providing regular review and assessment of performance against the quality policy.

Within the quality system, quality management and quality policy are implemented by quality planning (ie, the establishment of the quality objectives to be met for key elements of quality). This includes:

- safety, performance, stability, fitness for use, and the related requirements for quality (taking account of the stated and implied needs of the product users and all legal and other requirements of the society in which the company operates)
- quality control, which covers the operational techniques and activities that are needed to fulfill requirements for quality
- quality assurance, including all the planned and systematic activities necessary to provide adequate confidence that the requirements for quality are being fulfilled
- quality improvement, covering the actions taken to increase the effectiveness and efficiency of activities and processes to provide added benefits to both the organization and its customers.

In addition, all the elements, requirements, and procedures adopted by a company for its quality management system should be documented in a systematic and orderly manner in the form of a quality manual, quality plans, and quality records. The quality system applies to and interacts with all activities pertaining to the quality of a product and process and involves all phases from initial identification to final satisfaction of requirements and customer expectations. The system functions in such a manner as to provide proper confidence that all factors affecting the quality of products are under control, such control being primarily oriented toward prevention of quality deficiencies. It is also a management resource in the optimization and control of quality in relation to risk, cost, and benefit considerations.[33–37]

The ISO 9000 series of standards[33–37] is an attempt to rationalize and harmonize the diverse approaches in this area. Two types of standards are presented in this series. The first, ISO 9004,[37] provides guidelines for the development and implementation of an inhouse quality system and is designed primarily to satisfy the internal managerial need of a company for enhanced productivity, cost-effectiveness, and confidence in regard to fulfilling customers' expectations. The second is ISO 9001, ISO 9002, and ISO 9003,[34–36] which provide models of reference to be used in contractual situations. They identify the elements the quality system of the supplier or producer should cover as a minimum and serve as criteria for the formal assessment, recognition, and accreditation of the quality system, usually by a third party. Such models differ according to the functional activities to be developed by the supplier or producer to ensure that the requirements of the contract are being fulfilled: ISO 9001, when the activities may involve design, development, production, installation, and servicing; ISO 9002, when the activities cover production and servicing; ISO 9003, when the conformity with the specified requirements is to be ensured through final inspection and tests only.

Developing the requirements of the ISO standards goes far beyond the scope of this chapter, which considers the development of quality systems in relation to food safety and HACCP. A company accredited to an ISO 9000 standard has had to consider each paragraph of the standard and to fulfill its specified requirements; however, when dealing with such a company, one should be aware of the positive and negative aspects of ISO 9000 accreditation. The first relates to the fact that such standards delineate only a generic framework for an organizational approach. They do not identify the methods by which the identified activities should be carried out. This is beneficial in avoiding an excess of formalism with regard to accreditation, while allowing for flexibility to adapt to the practices of each industrial sector. It means that the management of a company has the responsibility for choosing the most appropriate and efficient method to be used with specific regard to the activity, the product, the process, and the quality attribute under consideration. The second refers to the choice of the standard. ISO 9003, limited to final inspection and testing, usually is considered as inappropriate to the food processing industry because of the limitation of end-product sampling and testing to provide appropriate confidence in products meeting their requirements for quality. ISO 9002, which covers production and servicing, generally is considered the most appropriate standard for the food manufacturing sector; however, a specific difficulty may arise from the choice of ISO 9002. Basically,

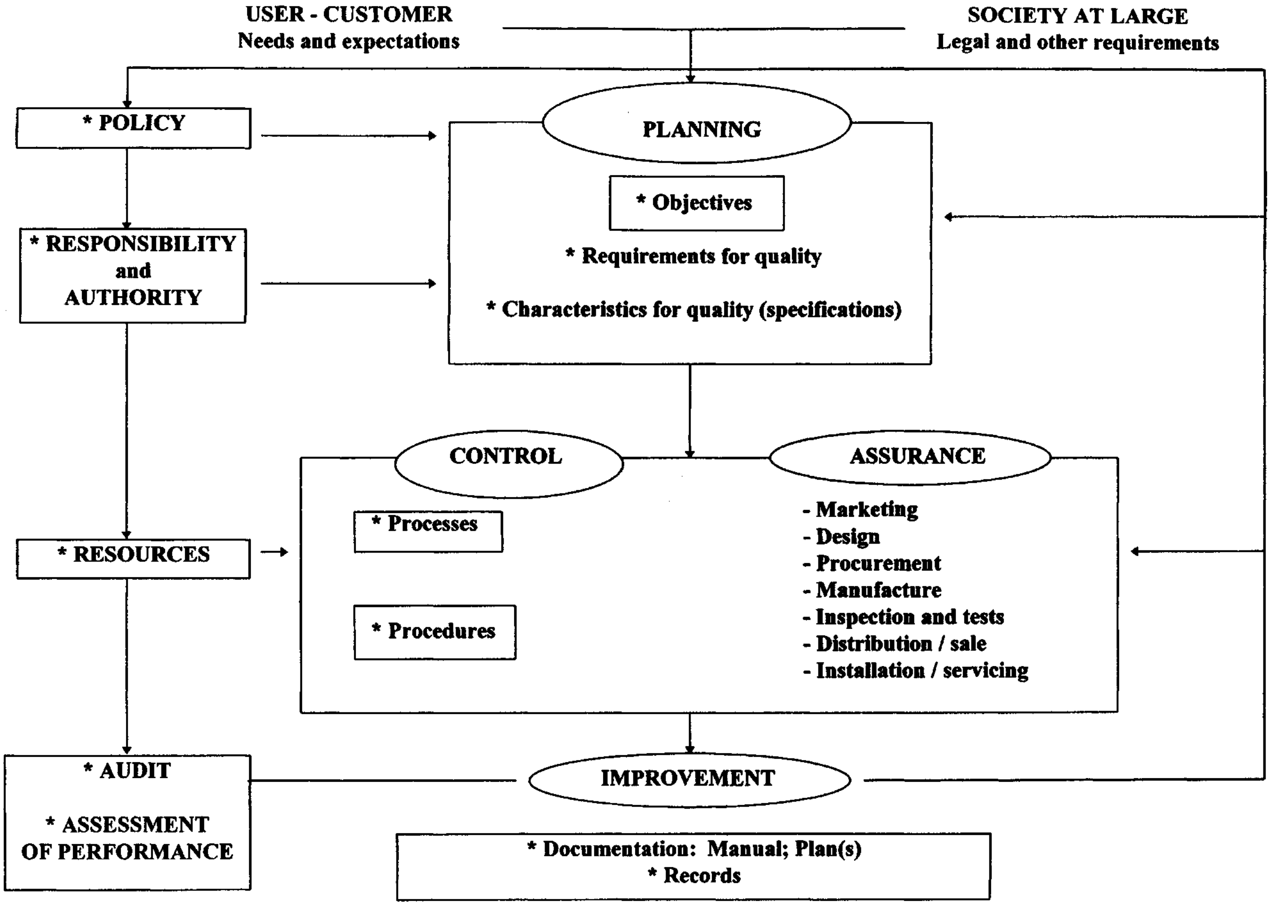

Figure 58–5 Fundamental Elements and Functions of a Quality System

this standard is to be used when the requirements for the product are expressed in the form of established or specified characteristics or specifications and when an appropriate confidence in the product meeting the stated requirements can be obtained by the demonstration of the manufacturer's ability during production.[35] This clearly means that the output will reflect only the requirements that have been stated. A possible consequence is that if such requirements have some deficiencies, or if they do not reflect all consumers' expectations, the product manufactured to such requirements will be deficient or will not fully meet consumers' expectations. Such considerations are crucial where safety is concerned. Safety starts at the product/process concept and design stages, and specifically requires proper hazard analysis and management decisions. The question, therefore, is how to introduce such considerations into a quality system, while identifying the most appropriate methods to do so.

The answer is simple: deal with safety matters by preparing a specific safety assurance plan based by priority on HACCP concepts and methodology. The concept of a "quality assurance plan" on which this approach is based refers to "a document setting out the specific quality practices, resources, and sequence of activities relevant to a particular product, contract or project" (ISO 8402). A quality assurance plan is an important tool. It identifies key elements in the product necessary to provide fitness for use and means by which they are provided and measured. With regard to such key elements, a quality assurance plan operating within the quality system is particularly useful in the following situations:

- where design (or redesign) is necessary or may be complex
- where all aspects of product/process design have not been fully assessed and validated
- where production is complex due to several factors such as availability of validated processes, number and diversity of operations, and high impact on product characteristics critical in regard to its performance.

The management of safety is a critical and complex issue in the food industry, which fits very well in the scope and field of application of a specific quality assurance plan. It is also where the use of HACCP as an effective tool has priority. An HACCP-based safety assurance plan specifically applies to a particular product and its process of production and assesses the activities specifically related to safety hazards. In contractual or regulatory situations, such a plan can be used to demonstrate that safety matters have been properly addressed and that appropriate control is being carried out. From a practical point of view, two situations are possible for the implementation of an HACCP plan.

The first occurs where no formal quality system exists. Here the HACCP approach assists in addressing many of the elements of the ISO standards with specific regard to safety.[38] When subsequently introducing an ISO 9000–based quality system, the safety assurance plan based on HACCP becomes a basic constituent of the quality system; in fact, a company following the 12 steps in applying HACCP will find itself well down the road to complying with ISO 9000 requirements.

The second occurs where there is a formal quality system in place. Here, the HACCP plan defines the specific application of the quality system to product safety. It may contain specific requirements with regard to safety that are more stringent than required by the overall quality system (eg, design validation in an ISO 9002 context) and otherwise refer to the elements of the quality system that are relevant to the product safety. In both cases, the safety assurance plan based on HACCP and the quality system are intimately associated and their integrated use provides additional benefits.[44]

In particular, the quality system facilitates the management of safety and use of HACCP in three ways. The first is that appropriate control of several activities can be established through the GMP (eg, sanitation, preventive maintenance, training of personnel), thus allowing the specific safety assurance (HACCP) plan to focus on specific and critical aspects in regard to safety (and, where appropriate "keepability"). The second is that the quality system addresses some elements that are not explicit components of HACCP, although important for its effective management. These include management responsibility and policy (provisions for safety and HACCP), quality system organization (establishment of a specific safety assurance plan), contract review, document control, product identification and traceability, internal quality audits, and training. The third is that ISO standards (ISO 9004 in particular)[37] provide organizational guidance to appropriately carry out specific activities that are components of HACCP. These would include design control (validation of product and process design with respect to safety), process control (specific process characteristics and CCPs), control of nonconforming products, corrective actions, and quality records.

Conversely, HACCP provides assurance that safety matters have been addressed properly and that appropriate control is established at all relevant stages of the product cycle (ie, design, purchasing, production, inspection and testing, handling, storage, delivery, servicing). Integration of GMP, HACCP, and a quality system such as ISO 9000 will deliver benefits to the food producer by providing greater certainty that foods marketed will be safe, and to the consumer that foods purchased will not be harmful to health.

58.6 CONCLUDING REMARKS

The consumer expects to receive foods that, as well as meeting expectations of quality, are safe. Meeting safety expectations is a core business aim, and meeting food safety requirements is part of the price of admission of a company's products to the marketplace.

Technical support is essential to the production, processing, packaging, and distribution of microbiologically safe foods. Underpinning research is of paramount importance to achieving such technical aims, and the chapters in this book and elsewhere[30,40] highlight microbiological research needs to further develop high-quality and safe foods. The use of properly formulated guides to good manufacturing and hygiene is a practical means of disseminating this knowledge to all food producers. Their development by food manufacturers and their trade associations should be continually encouraged by governmental agencies.

The modern approach to identifying microbiological safety requirements for a foodstuff is ideally based on a two-stage procedure (ie, the process of government-led risk analysis [risk assessment, risk management, and risk communication]) followed by the use of HACCP by industry to identify and manage the specific microbiological hazards associated with a particular food production operation. However, microbiological risk assessment is in its infancy, and we have much to learn before we can make scientifically-based quantitative assessments of microbiological risk; see Chapter 65.

The further development of HACCP requires more reliable means of identifying hazards and developing sound rules for establishing CCPs and critical limits; this may well be assisted by the further development of microbiological databases and predictive models of microbial growth, survival, and infectivity.

The development of risk analysis and HACCP will require scientists in academia, government, and industry working together closely. It is important to recognize the links between government and food industry activities in food safety assurance; such links are illustrated in Figure 58–6 and show the important interactions arising from these activities.

If the benefits of HACCP are to be applied most widely, and to benefit all food producers and consumers, it is important that the system be kept flexible in its application. It must be seen clearly as a tool for use by industry and not as a means of enforcing unnecessary controls on industry. The authorities' role should be one of encouragement and assistance in applying HACCP.

The food industry must organize itself to provide greater assurance that the procedures that are necessary to ensure microbiological safety are properly managed. It is essential that quality management systems are more widely used throughout the food industry and that these incorporate proper consideration of safety needs by the use of HACCP within the framework of a quality management system,

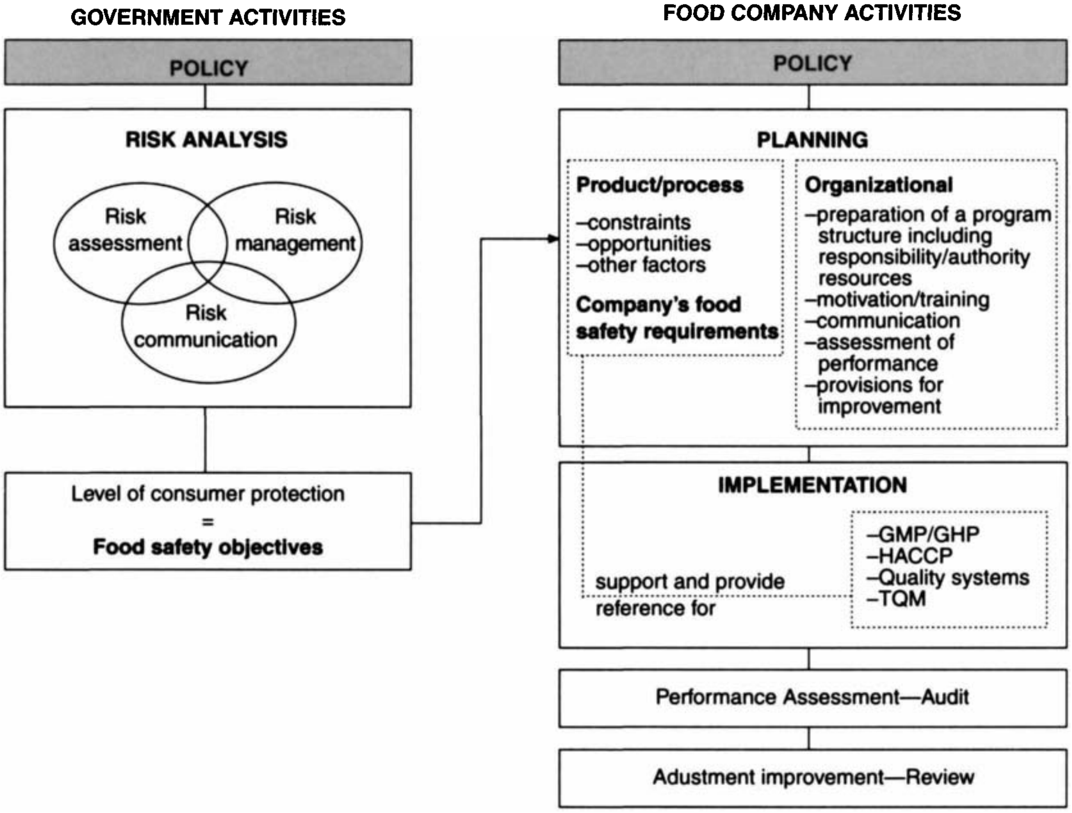

Figure 58–6 Interaction between the Government's and a Company's Food Safety Activities. *Source:* Reprinted with permission from ILSI Europe, *Food Safety Management Tools*, © 1998, International Life Science Institute.

such as ISO 9000. The increasing sophistication of many modern food production processes, and the greater use of milder conditions for processing foods, requires far greater control to be exercised over production procedures. Only companies that recognize what controls and operational practices need to be in place to market safe foods are likely to be successful in the marketplace in the 21st century.

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59 Hygienic Design of Factories and Equipment

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59.1 INTRODUCTION

Problems caused by excessive microbial contamination of foods tend to be expensive, particularly if they result in public recalls. As a result of the development and application of increasingly mild preservation technologies, such as ohmic heating, microwave heating, and the use of modified atmospheres and chilled storage (see Chapter 3), processed foods become more sensitive to microbial (re)contamination, requiring greater control of the manufacturing process.

The hygienic design of food factories and equipment plays an important role in controlling the microbiological safety and quality of the products made. This is officially recognized in legislation, such as the various directives of the European Commission (eg, the "Hygiene of Foodstuffs" directive[16] and the so-called "Machinery" directive),[15] which incorporates an annex relating to "Machinery for Agri-foodstuffs" and in the United States (US) (eg, the "Pasteurized Milk Ordinance"[24] and the Fish and Fishery Products Hazards and Control Guide).[25] The Codex Alimentarius[13] also addresses hygienic design in relation to food safety.

A hygienic factory should prevent products from:

- having too high microbial counts
- containing toxins of microbial origin
- containing residues of chemicals used for cleaning and disinfection
- being contaminated with other nonfood substances such as lubricants, coolants, and antimicrobial barrier fluids
- containing foreign bodies, such as pieces of metal, plastic, packing material, and insects (or other vermin) or parts thereof.

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High Microbial Counts. Too-high microbial counts may be the result of high counts in the raw materials or the survival of microorganisms due to failing of process control or equipment; too-high counts also may be caused by recontamination of product. A process also may fail because of inefficient mixing or too-short treatment times. Inefficient mixing may cause inhomogeneous temperature distribution, and the measured temperature may be different from the temperature of the bulk of the product and—most importantly—from the temperature of the coldest part of the product. Too-low temperatures in thermally processed food also may be the result of build-up of food or food-derived materials on heat-exchanging surfaces. A wrong design of the factory may result in mixing raw with decontaminated product or the transfer by other means (eg, air, personnel, or equipment) of microorganisms from raw to finished product ("cross contamination"). Equipment, correctly cleaned and sanitized before use, may be recontaminated by the product as a result of an incorrect startup procedure; the contaminated equipment may subsequently recontaminate passing product. Further, individual components of process lines, if of incorrect design, may cause recontamination. Incorrectly designed process lines, even if properly cleaned (eg, manually with disassembly where needed), may lead to contamination of product and thus to high microbial counts because microorganisms may multiply to high numbers in areas where product is stagnant ("dead" areas) and may release large numbers to passing product. Such areas often are found at the sides of conveyor belts and in pipelines, where appendages such as pressure gauges and temperature sensors have been mounted. A stagnant area that seems unavoidable is the rim of the filling nozzle, where there is always accumulation of product. A single microorganism may convert the accumulated product into a reservoir of spoilage or food poisoning microorganisms. The contaminated product will continually re-enter the product being filled, sometimes as small lumps or droplets, depending on product viscosity. The process equipment itself may still be contaminated after cleaning and sanitation as a result of not being cleanable (due to the presence of non-cleanable features such as screw threads and crevices), not being given the required treatment (because it is not resistant to the heat or bicidal chemicals), or just of not being properly cleaned because the cleaning procedure is incorrect or the control system does not function or has not been set up properly.

In an open plant, where product is exposed to the environment, microbial contamination may come from the air, dust, condensate falling down from chilled pipelines, splashes of other liquids, insects (or sometimes even birds), and directly from people touching the product. The risk of contamination is inversely proportional to the cleanliness of the plant and hence to its cleanability, which in turn depends on the design of the building and design and installation of all that is inside the building.

In closed process lines, the incorrect use of so-called "mixproof" valves may lead to recontamination. Fouling of the holding section in a continuous pasteurizer or sterilizer may significantly reduce the volume in that section, leading to a too-short residence time. If seals between components fail, product may leak to the outside of the line. Microorganisms may then multiply in the product and grow back to the product side. In the case of dynamic seals, such as those for shafts of valves, pumps, and mixers, the movements of the shaft will assist the transfer of product to the outside and of microorganisms to the product side. This applies to reciprocating shafts and to a lesser extent to rotating shafts, the latter always displaying some axial movement. Where it is necessary to prevent the ingress of live microorganisms, double seals with microbicidal barrier liquids are used. Such barriers, however, if not timely replaced, may become a growth medium for microorganisms.

Where microbial growth in the product is controlled by its composition, failing sensors or incorrectly calibrated measurement equipment may result in deviations in pH, water activity, and concentration of preserving agents.

The primary packaging material may be contaminated unacceptably, because the quality as delivered is not good enough or—in case of aseptic packaging—because of inadequate decontamination or of recontamination of the material.

Toxins of Microbial Origin. Microorganisms may produce toxins that survive heat, such as *Staphylococcus aureus* enterotoxin and many mycotoxins, such as aflatoxins, ochratoxins, trichothecens, fumonisins, and zearalenones. Cooking, pasteurization, and sterilization do not destroy such toxins; therefore, if a product—at any stage in the product chain—has acquired a too-high count of toxigenic microorganisms, that product should never be upgraded by destruction of the microorganisms, but should be destroyed to avoid it from being used.

Chemical Residues. Chemicals used for cleaning and disinfection of equipment or packing material should never be left behind to contaminate the product. The concentration of any residues should not exceed the legally accepted level. For instance, in many countries the product may not contain more than 0.5 mg hydrogen peroxide per kg product. Preferably, residues should be nontoxic. Intrinsically toxic products such as tetraborate and chloroisocyanuric acids should be avoided in food processing plants.

Construction materials (ie, metals, polymers, elastomers, but also auxillary substances such as lubricants, antimicrobial barrier liquids, coolants, and signal transfer fluids) may contain toxic substances that may migrate to the product, depending on product characteristics (such as pH and chloride contents). Therefore, materials used for the construction of components that may come in contact with food should be selected very carefully. Under the conditions of use, these materials should not be able to make a food product toxic.

Foreign Bodies. Foreign bodies may originate from raw materials (bones, insects) and packing material (wood, cardboard, metal, glass, polymers), but also from the building (glass, wood, chalk, paint flakes, screws, nails), equipment (bolts and nuts, security rings, pieces of gaskets, metal and polymer shavings), the environment (insects, rodent and bird droppings, dust), and people (jewels, pen, pencils, hair, hairpins, buttons, nails) and their activities (tools, bristles from brushes, pieces of cloth). The design of the plant should prevent foreign bodies from contaminating the product in all stages of the manufacturing process. The correct choice of construction materials; measures to prevent the presence of insects, rodents, and birds; and facilities for personnel to safely store their belongings and their tools will greatly reduce the risk of foreign body incidents.

Hygienic Design. A factory that has been designed with hygienic requirements in mind, process lines correctly built from hygienic equipment, and properly operated and maintained will produce food products that are safe and of excellent microbiological quality.

This chapter deals with the influence of the design of a plant on the quality and safety of the product and discusses hygienic requirements. Many pitfalls will be discussed, as will ways to avoid them. Examples of wrong designs and of designs complying with hygienic requirements will be given. Examples are just that; there may be other solutions, particularly because of the rapid developments in new construction materials and methods, for buildings as well as equipment.

A final remark: there are many types of food, and susceptibility to microbial attack varies widely. The only way to decide on which measures are needed is to carefully estimate the risk for each product and every single process step (ie, apply Hazard Analysis Critical Control Point) (see Chapter 58).

59.2 FACTORY DESIGN

59.2.1 Layout of Food Factories

The production plant must be built in such a way that finished product cannot be recontaminated by raw materials (including packaging materials) or any intermediate product. Thus, the flow of material should be from the raw materials reception to the separate finished product exit. Similarly, it should not be possible for personnel working with raw materials to move to or pass through upstream areas, where products may become contaminated. Stores should be located and provided with entrances and exits in such a way that to obtain clean materials, no less clean areas need to be crossed. Functions not directly involved in the manufacturing process, such as administrative offices, laboratories, and canteens, should be separated from the product areas to prevent staff from entering these areas. Preferably, they should be in a separate building. If not, they should be separated from the product areas by a corridor.

Figure 59–1 gives a generalized example of the layout of a food factory, assuming that there is a process step following the steps where the relevant microorganisms are inactivated, and not followed by another process step that would provide the required inactivation. Arrows indicate the (compulsory) direction of the flow of materials. Which areas are needed should follow from a hazard analysis, taking into account product vulnerability and shelf life requirements.

Reception and Storage Areas. The layout of the factory must be such that raw materials can be properly stored in the correct areas immediately on reception. Products presenting different hazards or with different vulnerabilities should not be stored together, nor should it be necessary to cross different storage areas. In the current example, dry, frozen, and chilled raw material areas all can be reached from the unloading bay. Also, the packing materials, chemicals, and spare part stores can be reached from that bay. Although liquid raw materials may be delivered in containers like barrels, drums, bottles, cans, or jerrycans, often they are delivered by tank

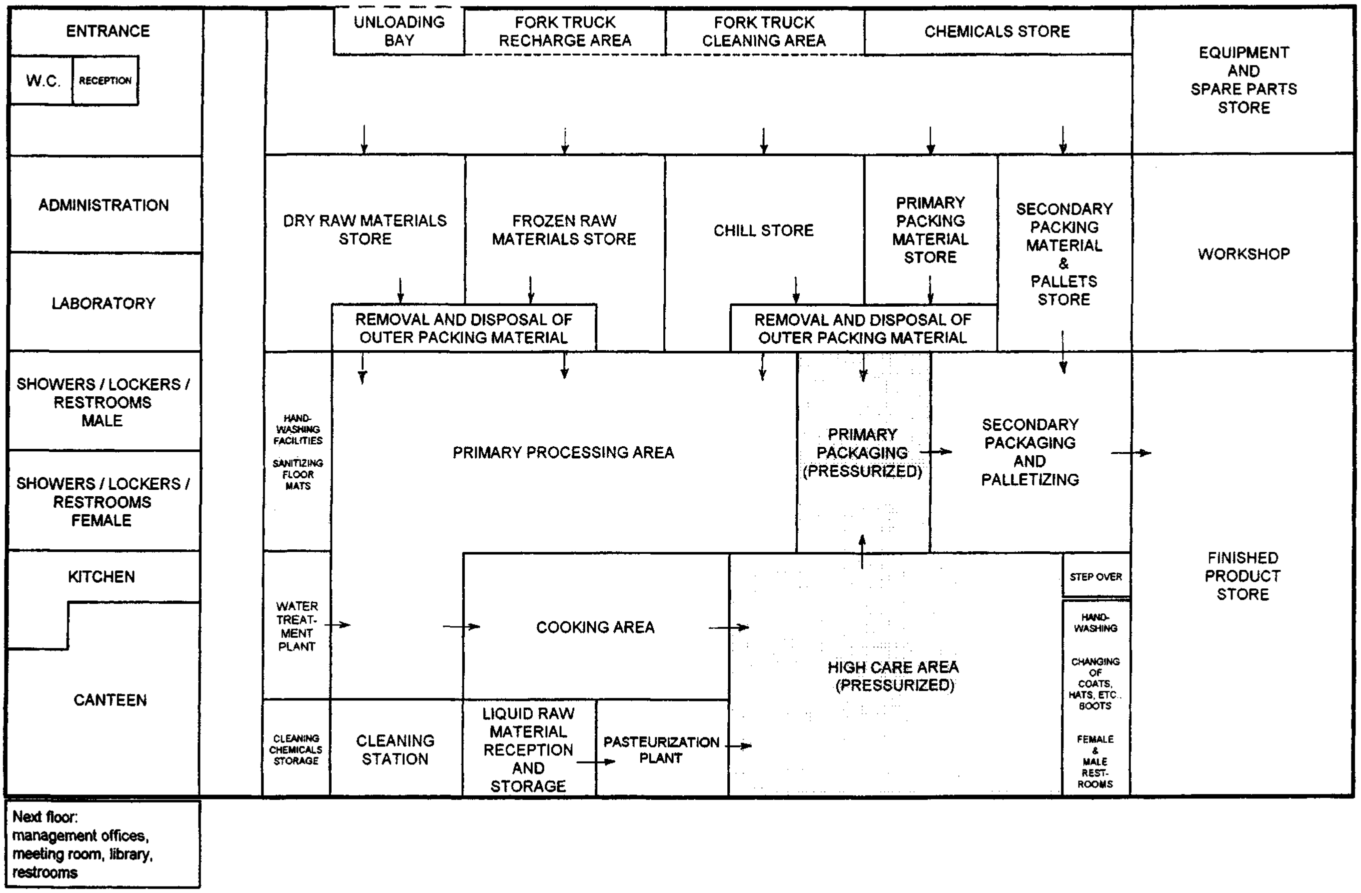
ENTRANCE
W.C.
RECEPTION
ADMINISTRATION
LABORATORY
SHOWERS / LOCKERS / RESTROOMS MALE
SHOWERS / LOCKERS / RESTROOMS FEMALE
KITCHEN
CANTEEN
Next floor: management offices, meeting room, library, restrooms
UNLOADING BAY
FORK TRUCK RECHARGE AREA
FORK TRUCK CLEANING AREA
CHEMICALS STORE
EQUIPMENT AND SPARE PARTS STORE
DRY RAW MATERIALS STORE
FROZEN RAW MATERIALS STORE
CHILL STORE
PRIMARY PACKING MATERIAL STORE
SECONDARY PACKING MATERIAL & PALLETS STORE
WORKSHOP
REMOVAL AND DISPOSAL OF OUTER PACKING MATERIAL
REMOVAL AND DISPOSAL OF OUTER PACKING MATERIAL
HAND-WASHING FACILITIES
SANITIZING FLOOR MATS
PRIMARY PROCESSING AREA
PRIMARY PACKAGING (PRESSURIZED)
SECONDARY PACKAGING AND PALLETIZING
FINISHED PRODUCT STORE
WATER TREAT-MENT PLANT
COOKING AREA
HIGH CARE AREA (PRESSURIZED)
STEP OVER
HAND-WASHING
CHANGING OF COATS, HATS, ETC., BOOTS
FEMALE & MALE REST-ROOMS
CLEANING CHEMICALS STORAGE
CLEANING STATION
LIQUID RAW MATERIAL RECEPTION AND STORAGE
PASTEURIZATION PLANT

Figure 59–1 General Layout of a Food Factory. Courtesy of Unilever Research Vlaardingen.

cars (road tankers), which is the case in the example where the liquid raw material reception is at a separate area, close to where the liquid is to be processed. The outlet valve of the road tanker is connected to the inlet valve of the reception area, which is mounted on the outside wall. The connection is cleaned and sanitized before the valves are opened to transfer the contents of the tank to the reception vessels inside. The product often is pasteurized or sterilized immediately on reception. It may be necessary, however, to keep the product cool for some time before heat treatment.

The relatively large quantities of cleaning and sanitation chemicals are delivered directly to the store next to the cleaning station. This station may host both the cleaning-in-place (CIP) installation and facilities for cleaning-out-of-place (COP), such as an industrial washing machine.

There can be many sources of water, such as municipal water, surface water, and well water. Whatever the source, often the quality needs upgrading, and a water treatment facility is common.[12] For convenience, the treatment plant is located close to where the water is needed most.

Processing and Packaging Areas. To prevent excess contamination before delivery to processing and primary packing areas, the outer packing materials must be removed in a separate space, which should have adequate facilities for dust-free disposal (eg, a well-vented compactor). Means for transporting raw materials to the processing and packing areas should stay in those areas.

In the example, after primary processing (which can be all kinds of processes, including cutting, shaping, dough preparation, mincing, sorting, and assembling), the product is passed to the cooking area. The cooked product must be protected against recontamination and, therefore, is transported by suitable means (often a conveyor belt or chain) to the high-care area, through a properly dimensioned slot in the separation wall. In the high care area, the final product is prepared, requiring the addition of the pasteurized liquid component (which could be a dairy-based product). From the high-care area, the product goes to the primary packing area, where the product receives its protection against recontamination. To reduce the risk of contamination while the product is not yet packed, the high-care area and the primary packing area are kept at a pressure above that in the adjacent areas.

From the primary packing area, the product is transferred to the secondary packaging and palletizing area and from there to the finished product store.

Workshop and Other Nonproduct Areas. To prevent undue risks of contamination, the workshop must have no direct access from any process area. There are connections only to the equipment and spare parts store and to the outside.

Administration, management offices, meeting room, library, change rooms, rest rooms, kitchen, and canteen are all separated from the production areas. The laboratory too should be separate, as in the example. That way laboratory staff can access all areas where product may have to be sampled (ie, all raw material stores and all production and packaging areas) without having to cross other areas.

Personnel can enter product areas only via disinfecting floor mats and a facility to wash, disinfect, and dry their hands. The high-care and the primary packing area can be accessed only via an area designed to reduce the risk of contamination of product in those areas. There is a step over, where only special footwear is allowed.

59.2.2 Outside Areas

Factory Surroundings. Preferably, food factories should not be located near areas where levels of microorganisms, insects, or rodents can be exceptionally high. Examples are farms, wastewater treatment plants, and municipal waste disposal areas. Nearby water (lakes, rivers, marshes, canals), although often desirable for other reasons (eg, transport, own water supply), is also highly undesirable from a hygiene point of view. This applies particularly to stagnant water. If the factory happens to be in the vicinity of such an area, extra measures may be required to achieve the hygienic conditions needed to produce safe food. Such measures may include fitting double external doors and the installation of additional air filtration plant.

The immediate environment of the factory should discourage pests entering or breeding. The environment therefore should be kept dry. If possible, the factory should be at an elevated level so that the soil outside will slope downward. Trees, shrubs, and flowers all attract insects and other animals and thus should be avoided in the immediate environment of the factory. Local specialists (biologists, entomologists) should be consulted about the effective distance between plant growth and the factory to prevent problems.

Often factories are illuminated at night. If this is the case, care should be taken that no insects are attracted. If possible, insect-attracting lights (high-UV lamps, mercury vapor lamps) should be positioned away from the building and lighting that is less attracting (using low-ultraviolet [UV] lamps, sodium vapor lamps) should be used if needed close to the factory building. Contrary to commercial claims, there is no evidence that ultrasound repels insects.

To keep pets and other animals away, it is recommended to install a fence around the factory. To be effective, the fence must be high enough and not of a design that serves as stairs or steps for animals. The fence must also extend deep enough into the soil to stop burrowing animals. The fence should be uninterrupted and any gate should be automatically closing.

Building Exterior. To deny birds places to breed, the outer walls of the factory should not have ridges or other protrusions. Where protrusions are unavoidable, measures must be taken to discourage birds from settling. The lower part of the wall should be rodent tight and the construction materials must be rodent proof. Rodents must also not be able to gain access to the inside by digging. The contractor should be required to guarantee that the building will be rodent proof. This applies particularly to the connection between the foundation and the external wall. Roofs, too, should be such that they do not attract vermin. The roof should therefore be kept dry and thus slope (>1%).

Air inlets must not be located near air outlets. The openings must be provided with screens to prevent insects and other pests from entering.

There should be no access for birds or insects to the space between the roof and a suspended "false" ceiling. Roofs and walls should be easily cleanable.

Doors and Windows. Doors and windows, if left open, allow the entrance of pests; therefore, doors should be self-closing. Doors and windows that are opened only rarely (eg, those for emergencies only) provide ideal places for insects to breed; therefore, care should be taken that doors and windows fit tightly, reducing any crevices between the door or window and its frame to a minimum. Depending on local conditions, it may be advisable to fit doors and windows with flexible strips; thresholds may be used. Where thresholds are not acceptable, other means should be used to seal between the floor and the door. There should be a program for regular inspection and cleaning of doors and windows. To avoid the attraction of birds, there should be no external window sills. If unavoidable, the windows should slope steeply without creating a breeding place for insects on the underside. Doors and frames should be made from (or treated with) rodent-resistant materials such as stainless steel. In areas where strong winds are common, doors should be located on the downwind side of the building. If windows are needed to provide daylight in the work area, glass bricks should be considered. They can be mounted flush with the wall and be maintained very easily.

59.2.3 Indoor Areas

To avoid cross contamination of products, the position of any air inlets and outlets should be carefully chosen. The air should move from the cleanest side or sides (where the finished products are) to the least (microbially) clean sides (where the raw materials are). This should be done correctly, which means for instance that the location of doors and windows should be taken into account. In some cases, doors must be interlocked so only one of them can be open at a time. To ensure that safety exits are not used as normal doors, they may have to be provided with alarms or be locked with a special key, which is kept behind a glass panel to be broken in case of emergencies.

All surfaces must be cleanable, which must be taken into account when selecting construction materials, and must be able to withstand the chosen cleaning methods, temperatures, and chemicals. Cleaning methods required usually are determined by the type of soil (including product residues) to be removed. Walls may have to be resistant to high-pressure jet cleaning and floors to mechanical cleaning with brushes; all surfaces may have to be resistant to chlorides and hypochlorite, hot alkali, and hot acid solutions. Decisions on cleaning methods should be made before, or in conjunction with, deciding on construction materials.

Floors. Floors must be smooth to ensure cleanability, they must not be slippery and they must be strong enough to withstand heavy duties such as the movement of forklift trucks. As far as known, there are no materials that meet all of these requirements completely, and the choice therefore usually will be a compromise.

Smooth concrete floors are normally fully acceptable for reception and raw material storage areas. In areas where product is not protected, ceramic tiles or resin floors are preferred. In high-care areas, seamless resin floors are the best choice.

If ceramic tiles are selected as flooring material, the supporting structure must be strong enough not to bend too much under the maximum possible load; otherwise, the tiles or grouting may crack and eventually the tiles may loosen. Loosening may be accelerated if the floor is moist; the presence of nutrients allows microorganisms to grow between and underneath the tiles, producing acid and dissolving the grouting.

If a resin is chosen as seamless flooring material, it must be certain that the resin can withstand all necessary and accidental treatments possible. This requires the material to be resistant to both cold and hot water, greases, cleaning and sanitizing agents, and to the spot pressure (pressure per unit area) caused by heavy machinery. The materials also must withstand abrasion caused by moving equipment such as trolleys. Resin floors should not be used in places of frequent use of forklift trucks.

Several types of resins (epoxy, metacrylate, polyester, or polyurethane) are available, and the selection must be based on the duty of the floor. The supplier should be able to guarantee the required chemical and wear resistance and the maximum spot load.

Any floor likely to become wet must have rounded corners where the floor meets the wall. If tiles are used, special tiles are available (Figure 59–2).

With the exception of areas that will always be dry, floors should slope toward drains or gutters. A slope of approximately 2% is an acceptable compro-

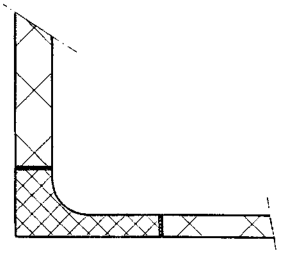

Figure 59–2 Tiles Avoiding Uncleanable Sharp Corners. Courtesy of Unilever Research Vlaardingen.

mise between the need for draining and the usual functions of a floor. In permanently wet areas, it will be hard to prevent eventual penetration of moisture, which will accumulate between the top floor and, in most cases, the concrete support floor. To protect chemical attack of the support floor, an impermeable bitumen layer (membrane) should be installed on top of the support floor.

Some flooring materials may be less slippery in combination with special footwear. The supplier should be able to advise and give guarantees.

Walls. Walls should be smooth and cleanable. They may be made from a variety of materials, such as concrete, bricks, or panels. Bricks and concrete should be covered by ceramic tiles or a good quality coating or paint. The choice of coating requires specialist advice as there are many coatings with widely varying chemical and thermal resistances, even within a type of coating, eg, epoxy, urethane, etc. The coating must be firmly fixed and sealed to prevent ingress of moisture. If tiles are chosen, they must be properly grouted to ensure they do not come loose during normal use, including cleaning. Expert advice (and guarantees) should be sought from specialist companies. Panels may be made from plastics, glass, aluminum, or stainless steel and often are sandwiched. In the case of sandwich panels, it must be ascertained that all sides comply with the chemical resistance required. Wooden panels should not be used in areas that may become wet. In dry areas they may be acceptable, provided the finishing is of good quality and the wood has been treated against the proliferation of insects and the areas are effectively kept free from rodents. It is important that all seams are reliably sealed. In some cases, seams can be sealed by welding with the parent material. In other cases, and where walls meet the floor, sealants should be used. The supplier should guarantee that the sealant gives a strong impervious bond and thus will not come loose from either the panel material or, where relevant, the flooring material.

Grouting. Normal cement grouting is hydrophilic and will absorb moisture and nutritious spillages, the presence of which may lead to microbial growth. Microorganisms often produce acids that may attack the grouting; therefore, in areas that may become wet, cement mixed with synthetic resins should be used as sealant between tiles.

Ceilings. Dust will always accumulate on the upper side of ceilings. Drafts cause pressure differences, which will move the dust through seams and openings for service lines in the ceiling; therefore, ceilings should always be hermetically sealed. Panels similar to those used for walls may be used. Lighting preferably should be built into the ceiling, the underside flush with the ceiling. If it is necessary to have lighting fittings at lower level, the upper side should slope sufficiently to avoid the accumulation of dust. The same applies to any other suspended items, including exposed beams. Ceilings and anything suspended from them must be easy to clean.

Doors and Windows. Requirements basically are similar as for exterior doors and windows. If windows need to be open, they must be provided with insect-proof screens. As some insects, often occurring in large numbers, are as small as 0.2 mm, the screens should have openings smaller than that.

59.2.4 Additional Requirements for Reception Areas and Raw Materials Storage Areas

Reception Areas. The area in which products are received should be designed to make it impossible for any vehicles to pass directly into the area where raw materials (including packing materials) are stored. Noticeboards are not sufficient: a physical barrier is essential. Unloading bays are very effective. As the doors to such bays will be opened for a prolonged time, the design of the bay must prevent the entrance of rodents. This can be achieved by using only smooth, vertical walls at the outside (ie, no stairs outside). Access of personnel should be by stairs inside the building only, or from inside the vehicle. To prevent birds and flying insects from entering, there should be flexible curtains or an effective air screen.

If vehicles must enter a storage area, they should pass an area where they can be cleaned before entering.

To prevent raw materials from contamination with dust, dirt, and insects, the layout of the reception area must be such that unloading is possible without placing the materials outside on the floor.

It must be possible to transfer vulnerable product that needs to be chilled or must be kept frozen to the chill or freeze storage area immediately. This must be done without opening the entrance to those areas for a significantly long time. If large quantities need to be unloaded into such areas, bays should be constructed that allow the unloading opening of the vehicle to be connected to, or for a short length, the vehicle to enter the area. This should be done in such a way that the clearance between the vehicle and the entrance is sealed sufficiently to prevent an unacceptable rise in temperature anywhere in the chill or freeze storage area.

Whatever way the product is unloaded, it must be possible to identify and if needed to label the incoming material and to take samples as needed from any batch of raw material delivered.

Storage Areas. All parts of the storage areas should be designed so that insects and vermin cannot hide and survive and that all surfaces are accessible for cleaning and inspection. There should be no deep recesses: supports of chests, cabinets, and scaffolding should be sealed to the floor or wall. The same applies to any appendages, such as lighting and switchboxes. Unless it is expected that the storage area will remain dry, surfaces should be nonabsorbent.

To prevent damage to the products stored and to prevent their contamination, the areas and constructions where the products are stored should be free from sharp protrusions, such as unprotected ends of beams, nails, bolts, and screws.

There must be separate areas for the storage of packing material, chemicals, spare parts of machinery, and tools. Access to the chemicals store should be restricted. All containers should be provided with a warning that they should not be in any production area during the production, with the exception of chemicals intended for use during production, such as disinfectants, lubricants, glue, and ink (in which case the storage in the production area must be limited to the amount needed for a reasonable production period).

There must be separate stores for intermediate and finished products. Personnel handling raw materials should not have direct access to the processing areas or the finished product stores.

Ventilation and Air Conditioning. If materials are stored for relatively long times (several days or more), the growth of microorganisms, particularly molds, may become a serious problem if the humidity in the storage area is higher than 55%. It is therefore important to keep the humidity below that value. Depending on local conditions, ventilation may be sufficient. If, however, microbiologically susceptible materials, including cardboard, which is used abundantly as packing material, are stored for more than a few days and the humidity in the environment is high, it may be necessary to install air conditioning or dehumidification equipment. In some cases, it may suffice to use conditioned air from the production area. Alternatively or in addition, the temperature in the relevant storage area may be reduced.

59.2.5 Additional Requirements for Production and Packaging Areas

Production areas may be divided into areas where product is exposed, the so-called "open plant" and areas where product is enclosed, called "closed plant." In open plants there is a higher risk of contamination of product from the environment. During primary packaging, the product is exposed and thus the area where product is packed should be treated as open plant, even if the product is delivered from a closed process line. Many requirements apply to both open and closed plants.

General Requirements for Open and Closed Plants. Basic requirements are similar to those discussed under reception areas and storage areas. Production areas should be designed so that vehicles coming from outside the factory cannot enter. Instead, materials should be transferred to vehicles dedicated to indoor transport.

All surfaces in the production area must be cleanable. Insect-proof screens with a sufficiently fine mesh should be used where open windows are used. There must be no niches or recesses where insects can hide. Equipment must be sealed to the floor or wall or enough clearance must be left for inspection and cleaning. Surfaces should be nonabsorbent unless only dry products are produced and the production area will always be dry.

It is strongly recommended to take all measures needed to prevent ingress of insects into areas where product or primary packing material is exposed, so that there will be no need for insect light traps. If insect light traps (with either electrocuter or adhesive surface board) are used, it must be made certain that trapped insects cannot contaminate food. They must not be mounted over exposed product or exposed surfaces (of machinery or packing materials) intended for product contact. The traps must be cleaned frequently to prevent other vermin from feeding on the dead bodies. It must also be understood that UV lamps used in the light trap may break and cause contamination of product with glass. Some manufacturers supply shatterproof lamps.

Gutters and Drains. Where possible, gutters (channel drains) should be avoided and hygienic floor drains (Figure 59–3) should be used instead. The cover of the drain should be flush with the floor. To reduce the risk of blocking with solids, the openings

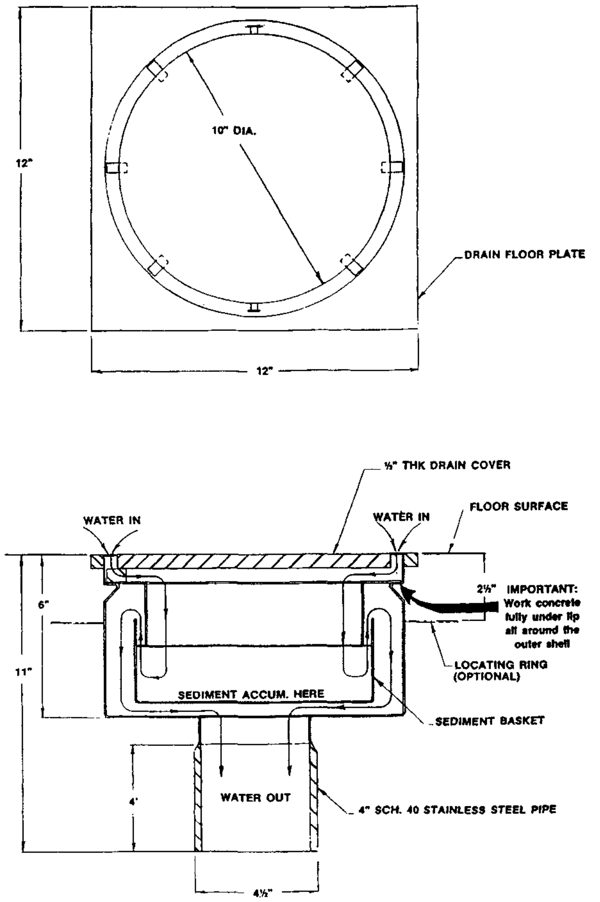

Figure 59–3 Example of a Hygienic Floor Drain. Courtesy of Kusel Equipment Co., Watertown, Wisconsin.

in the cover should preferably be tapered, the largest cross section at the underside. U-traps should be avoided as they accumulate dirt, providing a breeding place to insects. Floors should slope toward these drains. Floor drains must be cleanable. If they are placed in areas where the hygiene requirements are high, it may be useful to have drains that are suitable for the insertion of blocks or rings of disinfectant-releasing materials.

If gutters are necessary, they too should be designed to be easily cleanable. As they will not be covered all the time, they should be positioned near walls or other areas where there is no traffic.

Acoustic Absorption Materials. If the level of noise must be reduced by acoustic materials, care should be taken that these materials do not adversely affect the cleaning of the production area. Regrettably, sound absorption is best with porous materials, which cannot be cleaned. Moreover, for best effect they must be mounted some distance from walls or ceilings, creating places for nests of insects and other vermin, that are difficult to inspect and clean. Therefore, materials for hygienic applications have been developed that are somewhat less effective, but smooth and cleanable (by foam or mist). They can be mounted directly on the wall (Figure 59–4) or be suspended from the ceiling (Figure 59–5). In the latter case, cleaning may be more difficult and require (mobile) scaffolding or trucks with lifts.

Ventilation and Air Conditioning. Whether product is exposed or not, the risk of contamination from the environment will always be proportional to the concentration of microorganisms in that environment. It is therefore important that multiplication of microorganisms in the production area is prevented. As microbial growth is possible only in the presence of enough moisture, such growth can be prevented by ensuring a sufficiently low humidity. As molds will grow at water activities down to approximately 0.6, keeping the relative humidity at or below 50% is highly recommended. A humidity of 50% is also comfortable for human beings. Depending on the local environmental humidity, the installation of an air conditioning system may be required to keep the humidity down to 50%.

Water vapor may condense on cold surfaces, causing too-high water activities locally. Therefore, water vapor should be properly vented near to where it is generated and the installation of hoods may be needed. This may be the case where product is cooked, where vegetables are blanched, or where equipment or utensils are cleaned and sanitized using hot solutions or steam. The inside of such a hood should be inspected regularly for cleanliness as there

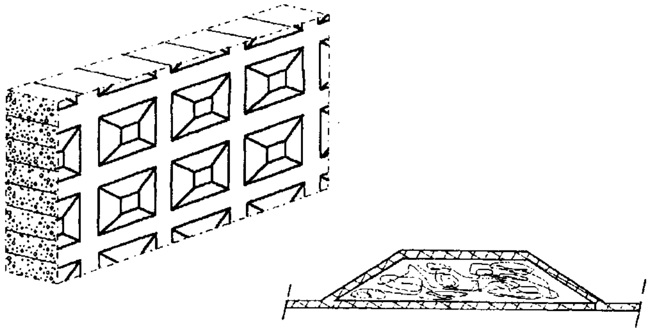

Figure 59–4 Sound Absorbing Panels with a Smooth Surface. Courtesy of Unilever Research Vlaardingen.

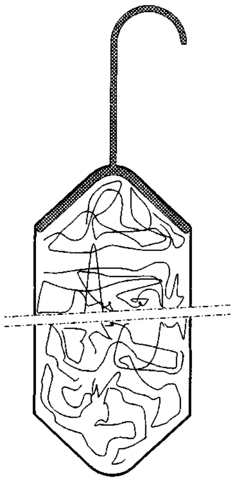

Figure 59–5 Smooth Acoustic Slabs for Hanging from the Ceiling. Courtesy of Unilever Research Vlaardingen.

the humidity will probably be high. It is likely that at least a part of the surface is at a temperature suitable for microbial growth, for at least part of the time.

Where dust is generated (eg, handling of dry powders, spices), care must be taken that the dust is extracted at that spot. For emptying bags, small or big, dust-free emptying systems are available, combined with dust-free packing material compactors.

Depending on local conditions, in particular the location of the factory (see Section 59.2.1), the microbiological product requirements, and the preservation processes used, it may be necessary to install air filters. See Section 59.3.3.

Dry Floors. Pools of water are ideal places for microorganisms to grow, in particular in food factories where the likelihood that sufficient nutrients are present is very high. The presence of pools of water and wet floors will also make it difficult to keep the humidity low. It therefore is strongly recommended to implement where possible a dry-floor policy (ie, a policy to keep floors dry at all times or, if wetting cannot always be avoided, to ensure that any moisture is removed as soon as possible). To achieve this, equipment should not be drained on the floor but into pipelines or gutters and there should be no hoses lying on the floor and spilling water, to be used in case product spillages must be chased away. Spillages should be collected and put aside in containers after which the floor is cleaned and dried. Water hoses should have self-closing nozzles and must be stored properly when not in use.

Thermal Insulation. If the outside temperature is low, even at a relative humidity below 50%, water may condense on cold surfaces, such as walls, doors, windows, and ceilings. If this is the case, the correct remedy is thermal insulation, preferably outside. If that is not possible and hence the insulation must be put on the inside, it must be made sure that it is completely isolated from the product area (eg, behind smooth and well-sealed panels, see Section 59.2.3).

Personal Hygiene. Hand-washing facilities should be installed near the entrance to the production area so that everybody entering the area passes these facilities. The taps should be operated without using hands (eg, by foot or elbow). Alternatively, electronic detection systems may be used. The facilities should include a provision of single-use (eg, paper) towels and cleanable waste bins. Rooms for changing clothing and footwear also should have adequate facilities for cleaning of hands and should provide locking facilities for personal belongings. Rest rooms (washrooms, toilets, flush lavatories) should be easy to clean and be provided with adequate means for cleaning, disinfection, and drying of hands. Preferably, operation of taps, dispensers, dryers, and doors should be automatic to minimize contamination risk.

In any area where direct or indirect contact with food is possible, nobody should be allowed access without a number of precautions aimed at avoiding product contamination risks. There are essential rules to prevent foreign body contamination:

- jewelry (including earrings and watches) should not be worn; they must be stored outside the production area in the lockers provided
- hair nets (also for facial hair) or effective caps or hats must be worn
- spectacles are allowed but must be tied to a necklace-string; preferably, the lenses should be made from nonglass material
- smoking and carrying cigarettes or any other smoking materials are not allowed
- eating, drinking, and carrying food, including sweets and snacks, are not allowed
- outdoor garments are not allowed and should be changed for clothing without buttons on the outside; continuous-thread polyamide zip-fasteners are preferred.

Other measures are aimed at preventing contamination with microorganisms:

- hands must be washed and, where product must be handled manually, disinfected on entry of any exposed product area
- hands also must be washed when leaving any rest room; having done so, it is still required to wash hands again on entering a process area, even if it means washing twice in a very short time
- shoes must be exchanged for shoes used only in the exposed product area; depending on the results of hazard analysis, it may be necessary to disinfect footwear on entering the food handling area
- factory clothing should not be taken home for cleaning, but left at the factory for professional cleaning at sufficiently high temperature to ensure inactivation of relevant microorganisms
- personnel must never move from less clean areas to cleaner areas without washing hands and changing factory clothing
- personnel with a health problem of microbial origin (or in case of doubt whether this is the case) should report to the company medical staff. When there is a risk of contamination of product, working in any exposed product or packing material area should not be allowed until free from infection.

Only production personnel and their responsible supervisors should have access to the food processing area. If technical staff, such as fitters, and professional visitors, such as food inspectors, must be given access, they should comply with all rules that apply to the area visited.

Packaging. During packaging, the product and often the product side of the packing material are exposed to the environment. The time of exposure, the concentration of microorganisms in the air, and the sedimentation rate of the microorganisms will determine the degree of infection of the product.

Even if packing is done aseptically, the rate of contamination of the packed product is influenced by the concentration of microorganisms in the surrounding of the packing machine. Depending on the acceptable contamination rate, the concentration of microorganisms in the area of the aseptic packing operation may have to be reduced.

59.2.6 High Care Areas

To keep contamination of exposed product under control, in addition to the measures that apply to all processing areas (see Section 59.2.5), extra measures may be required—which measures depend on the product and the intended storage, distribution, and shelf life. The measures may include restricted access, facilities to clean footwear, an area to change footwear and/or garments, maintenance of a positive pressure in the production area, and microfiltration of the air supplied to the area. A proper risk assessment should be carried out to establish which measures are needed to meet the targets set. Measures may include:

- a change area with a stepover that allows the exchange of footwear without contaminating the clean side with footwear from the other side
- supply of filtered air
- maintenance of a higher pressure to reduce ingress of contaminated air
- the use of gloves. Care should be taken that gloves are used properly. If not, they will increase rather than decrease the risk of product contamination. When using gloves to protect the product, no unclean surfaces should be touched. Gloves must be replaced frequently. As they promote the growth of microorganisms on the skin, care must be taken that these microorganisms cannot contaminate the food product; therefore, changing gloves should be done well away from the product (or primary packing material)
- mouth masks may be required; they usually are effective, provided they are replaced often enough
- if multiplication of microorganisms must be prevented during the processing in the high care area, which often is the case with meat products, the high care area should be chilled, typically to a temperature below 12° C. The actual temperature will depend on the possible rate of microbial growth in the product and the time the product resides in the area. Unless the residence time in the packaging areas is very short, these areas too must be cooled. Adequate clothing, which protects against the cold, must be provided. The clothing nevertheless must comply with the hygiene requirements listed above. For further information on hygiene requirements for high care areas, see the literature.[11]

59.3 EQUIPMENT DESIGN

The function of equipment is to convert raw materials into wholesome food products. If equipment is not designed correctly or assembled in the wrong

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way, the wholesomeness of the food may be impaired. Equipment may be the source of microbiological problems for a number of reasons. It may be that the equipment:

- cannot be cleaned and decontaminated and allows microbial growth, causing contamination of product in contact with the equipment
- should decontaminate (pasteurize or sterilize) the product but fails to do so
- although being cleanable, is such that part of the product resides in certain areas for too long a time, giving microorganisms time to multiply
- does not give the required protection against recontamination from the environment.

59.3.1 General Requirements

Materials of Construction

Materials intended for contact with food must be corrosion resistant, nontoxic, mechanically stable, and have a surface finish that is not adversely affected under conditions of use.[17]

Toxicity. No toxic construction materials should be used and the relevant legislation must be respected. The traditional stainless steel materials, such as types AISI-304, AISI-316, and AISI-316L, are fully acceptable for most applications (ie, under the conditions that these materials are corrosion resistant). Care must be taken when polymer and elastomer materials are used as they may contain leachable toxic components. The same applies to the use of adhesives, lubricants, and signal transfer liquids. Greases and lubrication oils should comply with §178.3570 of the US Food Drugs Cosmetics Law Reports.[26] The supplier must present evidence that the material is safe for use in contact with food.

Surface Roughness. Product contact surfaces should be smooth enough to be easily cleanable. The roughness (or smoothness) of a surface usually is expressed in μm, as R_a- value.[33] A surface finish of R_a = 0.8 μm is recommended.[1,17] To achieve this quality of surface, polishing or other surface treatment may be required. Cold rolled stainless steel sheet material, used for vessels and for piping, usually has an R_a-value between 0.2 and 0.5 μm and treatment thus is not needed. The surfaces should also be free from imperfections such as pits, folds, and crevices.[17]

Corrosion. To retain the desired smoothness of the surface, the equipment must be resistant to the product under the process conditions and withstand the cleaning procedure (concentration of chemicals at temperature of use for the times needed [eg,. 250 mg/kg sodium hypochlorite at 40° C for 20 minutes]) during the intended lifetime. Corrosion can be minimized by sticking to the concentrations, times, and temperatures specified, and by complete removal of residues (or neutralization if any is left at nonproduct contact surfaces of equipment). Many food products contain chloride and have a pH between 3 and 5, a very corrosive combination. Because in addition contact with product is much longer than contact with cleaning and sanitizing agents, often the rate of corrosion is determined by the product. This should be taken into consideration when selecting materials of construction.

The corrosion resistance of stainless steel is provided for a large part by a thin but effective film of chromium oxide on the surface. If the film is damaged, mechanically or chemically, corrosion of the iron under the film starts. The lifetime of stainless steel can be extended substantially by ensuring that any damage to the chromium oxide film is repaired, which is possible by treatment with oxidizing acids, such as nitric acid. This treatment is called "passivation."[41]

Welds. In principle, for permanent joints welding is the best method. A prerequisite is that welding is done correctly, which requires skilled welders. Not all welding methods can deliver welds of hygienic quality, but typically tungsten-inert-gas welding can. To obtain a good weld, materials must match (in composition *and* in dimensions), and during welding the materials must be fully protected by inert gas and the welding temperature must be correct. Otherwise, welds may seem correct when fresh and polished but will corrode rapidly in use. Bad welds can cause severe problems; therefore, to ensure that welds meet hygienic requirements, it is recommended to follow the European Hygienic Equipment Design Group (EHEDG) guidelines for welding of stainless steel.[22]

Gasket Materials. Traditionally, gaskets are made from rubber and particularly from synthetic rubbers or elastomers. Gaskets used in the food industry differ from those in other industries, in particular in that the gaskets often must be able to withstand a variety of conditions, such as subzero temperatures during processing and above 100° C during sterilization. Moreover, they must withstand a variety of products, such as acid and alkaline solutions as well as oils, at low and high temperatures. Gasket materials may easily impair cleaning and sanitation of equipment. To circumvent these problems, and because of both temperature and corrosion resistance, equipment manufacturers tend to use polytetrafluoroethylene (PTFE) as gasket material in food processing equipment. PTFE, however, lacks resilience. Due to the large difference in thermal expansion coefficient between PTFE and stainless steel, a heat treatment changes the shape of the PTFE gasket and, after cooling down, a crevice occurs (Figure 59–6). For a gasket of 5 mm thickness and a temperature change from 20 to 120° C and back, the crevice may be 36 μm wide (assuming no resilience at all; in practice, the gap will be slightly smaller). It is therefore strongly recommended not to use seals made from nonresilient materials.[39]

In 1996, gaskets and couplings (Figure 59–7) were designed to meet all requirements; they provide a reliable seal without affecting cleanability.[4]

Cleanability

To be cleaned without difficulty, surfaces must be smooth and free from crevices, sharp corners, protrusions, and shadow zones, not only when new, but during the entire functional lifetime.

Influence of Surface Roughness. Although good cleanability is the objective and smoothness is not, for practical reasons usually a maximum roughness is specified for food contact surfaces. Generally, cleaning time required increases with surface roughness.[48] Both the American 3-A organization and the EHEDG specify for food-contact surfaces a maximum roughness of $R_a = 0.8\ \mu m$.[1,17] According to the EHEDG, rougher surfaces can be acceptable if tests have shown that the required cleanability is achieved.[17] Porous surfaces usually are unacceptable.

Crevices. Crevices cannot be cleaned and will therefore retain product residues, which may effectively protect microorganisms against inactivation. In some cases, crevices are unavoidable. This may be the case if slide bearings are needed in contact with product (eg, as bottom bearings of top driven stirrers and as bearings in scraped surface heat exchangers). Their presence should be taken into account when writing procedures for cleaning and disinfection. These procedures may need instructions for partly or total dismantling of equipment or increased cleaning times. In most cases, crevices are the result of incorrect choices when designing (or choosing) equipment. When parts of equipment must be mounted together, metal-to-metal contacts (other than welds) must be avoided as they leave very narrow and deep crevices. Elastomers should be used between metal components but not in the form of O-rings in standard O-ring grooves, as this too will create crevices. The elastomeric material must be mounted in such a way that the seal is at the product side and that, to prevent destruction of the elastomer, excessive compression is prevented. This can be achieved by including design features that align the surfaces of the various parts and provide a metal stop. Figures 59–8 and 59–9 show examples of correct constructions.

Shadow Zones. There is much less transfer of energy to the food residues (soil) in zones in process equipment that are outside of the main flow of cleaning liquids than to the soil in the main flow. Such areas are difficult to clean and should be avoided. If unavoidable, their presence should be taken into account when devising the cleaning procedures. Typical shadow zones are the legs of T-pieces in pipelines used to mount sensors such as pressure gauges and products dividers/directors on conveyor belts.

Cleanability Testing. In case of doubt, the cleanability of equipment may be tested. Currently in Europe there are about 10 independent laboratories[10] that can test the cleanability of relatively small size equipment for closed processing (such as pumps, valves, and in-line sensors), using the EHEDG comparative cleanability test.[31] In this method, all product contact surfaces of the equipment to be tested and of a reference pipe are soiled with buttermilk mixed with spores of a *Bacillus stearothermophilus* strain as an indicator of removal

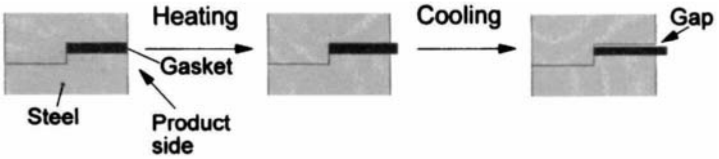

Figure 59–6 Development of a Microbial Leak due to Differences in Thermal Expansion between Stainless Steel and Nonresilient Gasket Material. Courtesy of Unilever Research Vlaardingen.

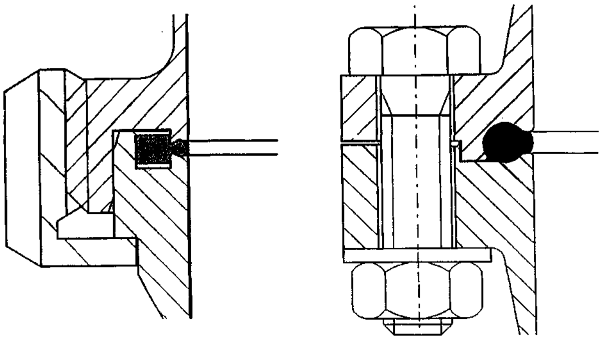

Figure 59–7 Hygienic Couplings Matching All Critical Design Parameters. *Source:* Reprinted with permission from F. Baumbach, J.P. Dubois, W. Grell, et al, Hygienic Pipe Couplings, *EHEDG Document 16*, as summarized in *Trends in Food Science Technology*, Vol. 8, pp. 88–92, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

of the soil. Then equipment and pipe are subjected to a very mild cleaning procedure so that some soil is left behind in the reference pipe. The number of spores still present after cleaning is a measure of the amount of soil left. The amounts left in the equipment tested and the reference pipe are compared. The method tells the equipment manufacturer whether the equipment tested can be cleaned more easily, more difficultly, or approximately as easily as a straight pipe.

A new method has become available, which is easier to apply, gives results faster, but is less sensitive than the above method. The method also is cheap and may be appropriate in many instances and certainly suitable as a means of preselection, reducing the number of the more expensive microbiological tests required during the development of new food processing equipment. The difference with the microbiological method is that instead of a bacterial spore, a colorant (β-carotene) is used as soil residue indicator.[52]

A consortium of laboratories and industries is developing methods to test the cleanability of larger pieces of equipment, such as homogenizers and scraped surface heat exchangers and very large pieces of equipment, for both closed- and open-process equipment.[32]

Decontamination of Equipment

Protection of Microorganisms by Soil Residues. Microorganisms can be very well protected by soil residues from destruction by heat. Sterilization with steam is dramatically impaired by the presence of soiled crevices.[40] For instance, treatment with saturated steam at a temperature of 120° C for 30 minutes will inactivate all viable *Bacillus subtilis* B1–12 spores on the surface of clean equipment, the D_{120}-value of these spores being approximately 14 s.[45] In soils consisting of normal food constituents, such as protein, sugar, fat, or a mixture of fat and protein, the D_{120} values found were 13 to 28

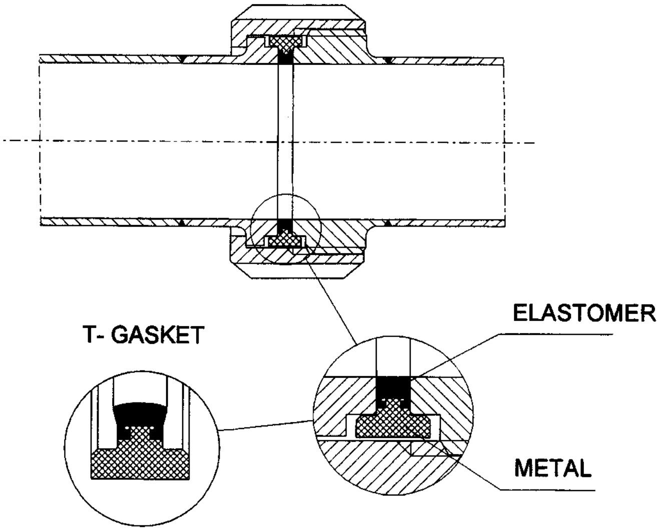

Figure 59–8 The ISO2853 Pipe Coupling, in Combination with a T-shaped Gasket as Shown, Ensures Correct Alignment of the Pipes and Controlled Compression of the Elastomeric Seal. There Are No Crevices. *Source:* Reprinted with permission from G.J. Curiel, G. Hauser, P. Peschel, et al, Hygienic Design of Closed Equipment for the Processing of Liquid Food, *EHEDG Document 10*, as summarized in *Trends in Food Science Technology*, Vol. 4, pp. 375–379, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

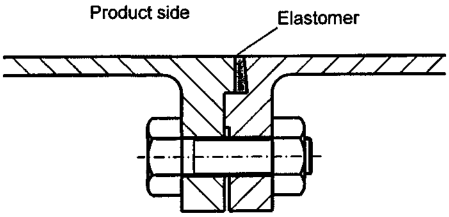

Figure 59–9 Principles of Joining Food Contact Surfaces so that Crevices and Ridges Are Avoided. *Source:* Reprinted with permission from G.J. Curiel, G. Hauser, D.A. Temperley, 1995, Hygienic Design of Equipment for Open Processing, *EHEDG Document 13*, as summarized in *Trends in Food Science Technology*, Vol. 13, pp. 305–310, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

minutes, at water activities of 0.5 to 0.6. Further, the reduction in number of viable spores in the protein/fat mixture dropped to a factor of 1,000 in a crevice at a distance of 15 mm from the surface. The decrease in degree of inactivation cannot be attributed solely to the slightly lower temperature in the crevice (approximately 117° C, for more than 10 minutes). More important seems to be that the water activity in the soil remains low despite the saturated steam passing the entrance of the crevice. D-values increase dramatically with decrease in water activity.[14] This applies to all types of microorganisms, including aerobic and anaerobic bacterial spores,[14] vegetative bacteria such as staphylococci,[54] and molds such as *Aspergillus* species.[20] Thus crevices may have a very dramatic effect on the sterilization of food processing equipment and hence on product safety. Clearly, it is essential that equipment is clean before decontamination.

Chemical Decontamination of Equipment. Open-plant equipment usually is decontaminated using microbicidal chemicals such as sodium hypochlorite, typically in a concentration of 50–200 mg kg^{-1} (total elemental Cl) at near ambient temperatures for 1 to 10 minutes, and quaternary ammonium compounds at 100 to 200 mg kg^{-1}. To save energy, often closed plants too are disinfected chemically.

Most disinfectants react with soil and become ineffective. It has been observed that chemicals hardly penetrate into crevices of the dimensions discussed above within the time usually allowed for the disinfection of equipment. The addition of surface active compounds does not improve the penetration. Apparently, diffusion is the only mechanism of transport of the disinfectant and this takes too much time. Chemical inactivation of microorganisms in soil present in crevices is simply not possible therefore. This has been confirmed experimentally.[6] This again demonstrates that food contact equipment must be free from crevices. Where crevices cannot be avoided, dismantling to remove soil prior to chemical decontamination is essential.

If closed equipment has to be suitable for decontamination with microbicidal solutions, there should be no air traps (dead areas), such as upwardly pointing legs, which would hamper the solutions to reach all surfaces. Air traps are often found near valves, temperature probes, and pressure gauges. Figure 59–10 shows such an air (and product) trap formed by the incorrect positioning of a pressure gauge on a T-piece. Figure 59–11 shows two layouts of valves and associated pipework for the transfer of product under sterile conditions to an aseptic tank and to protect the product in the tank from contamination while a product line is unsterile. Figure 59–11A shows a common layout of a valve arrangement with large traps, which may be a source of sterility problems; for details see "Sterility Barriers."

To ensure contact of chemical solutions with the inside surfaces of hinges, slide bearings, and dynamic seals (of any type), movable components should be actuated: conveyor chains should run, valves should be opened and closed, and pumps and stirrers should be switched on. For how many times and how long may have to be determined experimentally. The necessary actions must be included in the cleaning and disinfection procedures.

Thermal Decontamination of Equipment. Closed process lines are usually decontaminated by

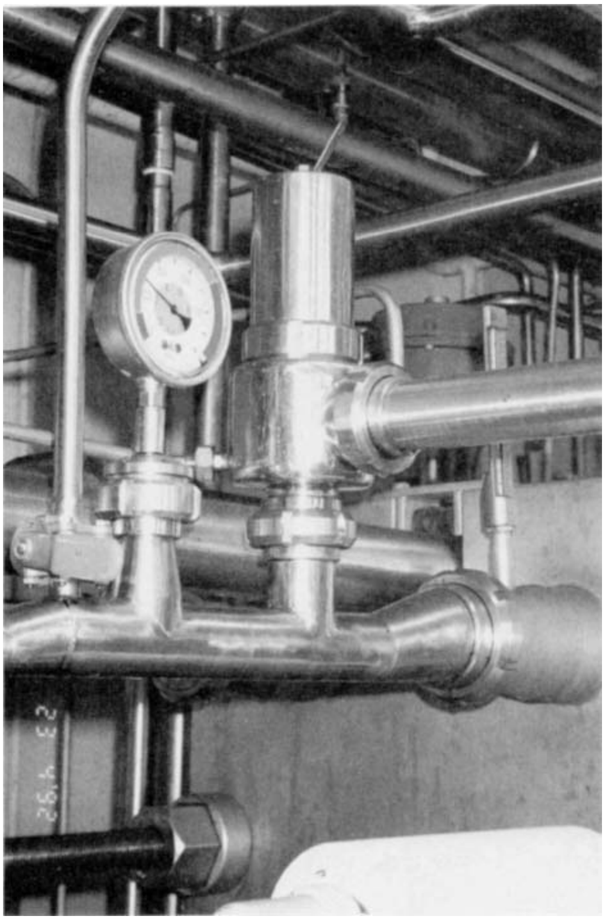

Figure 59–10 Traditional Way of Mounting Pressure Gauges and Safety Valves. Courtesy of Unilever Research Vlaardingen.

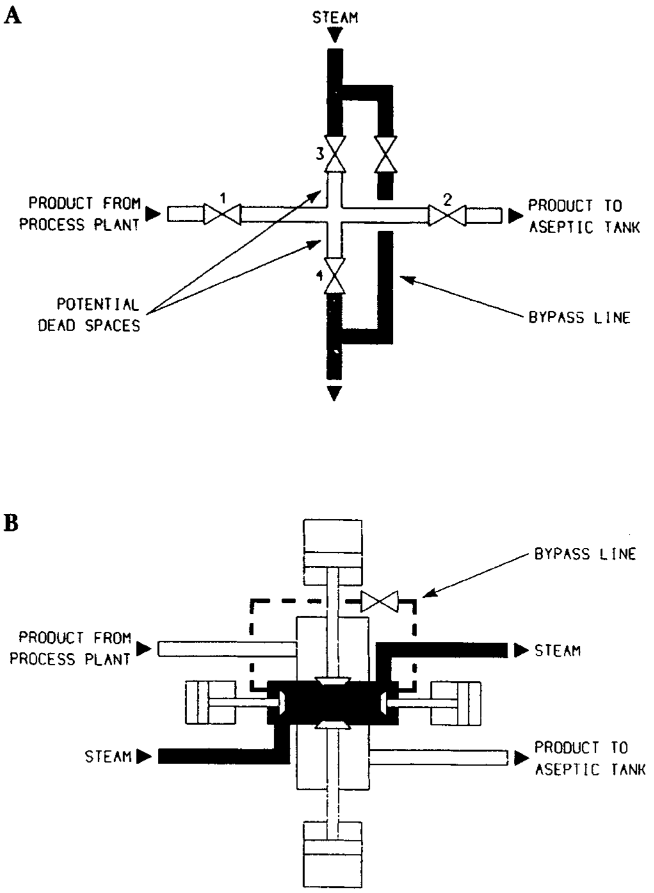

Figure 59–11 Two Designs of "Sterile Barriers." *Source:* Reprinted with permission from A.P.M. Hasting, S.A. Davies, and M. Lalande, The Continuous or Semicontinuous Flow Treatment of Particulate Foods, *Trends in Food Science Technology*, Vol. 5, pp. 88–95, © 1994, Elsevier Applied Science.

thermal methods. If bacterial spores are not relevant (eg, the line is used for pasteurized products), hot water is used. If bacterial spores are relevant, decontamination is done by either steam or hot water (under pressure).

Protection of Microorganisms against Thermal Inactivation by O-rings. Elastomers expand more than 10 times as much as stainless steel. The result is that heating causes an O-ring in an O-ring groove to expand more than the stainless steel by which it is enclosed. The contact surface between the O-ring and the steel increases with the rise in temperature and gradually more microorganisms are trapped between the rubber and the steel.[40] The O-rings thus protect microorganisms in a very similar way to soil residues; the water activity will be lower than intended, resulting in survival.[44] Obviously, the use of O-rings in food processing equipment increases product safety risks and therefore should, in principle, be avoided, unless special precautions are taken. For instance, O-rings of accurate dimensions may be used in equally accurate grooves so that entrapment of microorganisms is prevented and the ring is protected against excessive compression.[4] In many cases, the use of flat gaskets is easier.

Testing Whether Equipment Is Suitable for Thermal Decontamination. The EHEDG has defined standard methods to test the pasteurizability[51] and sterilizability[49] of equipment. These methods should be used if there is doubt whether a piece of equipment that must be free from (relevant) microorganisms before use can be pasteurized or sterilized. In these methods, the equipment to be tested is contaminated with a test organism (ascospores of *Neosartoria fischeri* for pasteurization and *Bacillus subtilis* spores for sterilization). Then the equipment is pasteurized (with water of 90° C for 30 minutes) or sterilized (with saturated steam of 120° C for 30 minutes). Subsequently the equipment is filled, aseptically, with nutrient medium to check for growth of the test organisms.

Preventing Contamination of Product with Liquids Used for Cleaning and Sanitation

Usually it is unavoidable to use chemicals for cleaning and disinfection of food processing equipment. Further, chemicals may be used to destroy unwanted microorganisms. Chemicals used may pose a danger to the safety and quality of the product to be made, however. It is therefore essential that all chemicals used are removed from the equipment before the production is started unless chemicals (such as peracetic acid) leave nontoxic residues.

Drainability of Equipment and Process Lines

To make it possible to remove all chemicals from process equipment, the equipment must be designed to be self-drainable[15]; thus, surfaces and pipes should not be completely horizontal but slope toward drain points and there should be no ridges that may hamper draining. Where it is not possible to build equipment so that proper draining is possible, procedures must be designed to ensure that residues of cleaning and disinfection liquids can be removed in another way. The method used should be well documented and there must be clear instructions. Draining is also important if no chemicals are used because many microorganisms can easily grow in residual water, needing only very minute amounts of nutrients.

Microbial Growth and Process Equipment

If the product is suitable for the multiplication of microorganisms and if microorganisms are present, multiplication will almost always take place, often even if seemingly all possible measures have been taken to prevent multiplication.

Residence Time Distribution. The geometry of equipment influences residence time distribution and there usually are places in process lines, even if correctly designed, where some product is residing longer than desirable. Even if dead areas have been designed out, there is always an amount of product attached to equipment surfaces, even at high liquid velocities. Microorganisms trapped may reside on such surfaces long enough to multiply. With an increase in the number of microorganisms, the numbers washed away with the product passing increase as well.

Biofilms. Depending on the properties of the surface and the types of microorganisms present, with time biofilms may develop that are very hard to remove. Biofilm development may take place on any type of surface and is difficult to prevent if the conditions sustain the multiplication of microorganisms. Many microorganisms, including many pathogens (*Listeria monocytogenes*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, and *Legionella pneumophia*) form biofilms, even under hostile conditions such as the presence of disinfectants. Actually, adverse conditions seem to stimulate microorganisms to grow in biofilms.[56,57] Thermophilic bacteria (such as *Streptococcus thermophilus*) can form biofilms in the cooling section of milk pasteurizers, sometimes within 5 hours, resulting in massive infection (up to 10^6 cells ml^{-1}) of the pasteurized product.[21,36] Biofilms shed microorganisms into the passing product. On metal (including stainless steel) surfaces, biofilms may also enhance corrosion, which may result in microscopic holes. Such pinholes are known to allow the passage of microorganisms present in the cooling medium and thus may cause infection of the product. Like other causes of fouling, biofilms will affect heat transfer in heat exchangers. On temperature probes, biofilms may seriously affect the heat transfer and thereby the accuracy of the measurement. On conveyer belts and on the surfaces of blanching equipment, biofilms may infect cooked or washed products, which are assumed to have been made pathogen-free by the temperature treatment received. Biofilms may be much harder to remove than ordinary soil. If the cleaning procedure is not capable of completely removing biofilms that may have developed, decontamination of the surface by either heat or chemicals may fail as biofilms dramatically increase the resistance of the embedded microorganisms. It thus is imperative that product contact surfaces are well cleaned before disinfection. Wirtanen[58] gives an excellent overview of fouling in relation to equipment for the processing of food.

Temperature Gradients. In attempting to prevent the growth of microorganisms in process lines by cooling, one should be aware that at all places where there is activity, energy is converted into heat, causing rises in temperature. The consequence is, that in chilled rooms (of any size) and in pipelines with cooled products, there are spots of higher temperature, where microorganisms multiply and subsequently infect passing product. Typical examples are mechanical seals, where the friction between the static and the rotating part dissipates a considerable amount of energy, and shafts of electromotors, which conduct the heat of the electric drive to the device in contact with the product. The device can be a fan, agitator, drive shaft of a conveyor chain, or rotor of a centrifugal or lobe pump. These effects can be reduced significantly by using barriers made from materials with a low thermal conductivity (eg, polymers or elastomer flanges between the motor and the driven piece of equipment). Cooling may also be applied (eg, in mechanical seals).

Other sources of heat are lights in freezer tunnels and cooling cabinets or any other electric instrument. Usually, it is possible to mount the heat generating part of the light armature outside the cold area, where adequate cooling can also be applied.

Quantification. The subject of growth of microorganisms in process lines has been given considerable attention as it may cause problems of which the origin often is not recognized. The potential importance can be illustrated by calculating the infection of a product as a result of the presence of stagnating product.

If a cell of *Escherichia coli* is trapped in a dead space filled with 5 ml of a slightly viscous low-acid food product at a temperature of approximately 25° C, it may take less than 24 hours for the number of *E. coli* cells to increase to a concentration of 0.2×10^9 ml^{-1}, assuming a doubling time of 40 minutes. If every hour just 1 ml is washed out from the dead space by the passing product, by the end of the first day of production, every hour the product is infected with 200 million *E. coli* cells. If the production capacity of the line is 5 million ml h^{-1}, the average *E. coli* contamination will be 200/5 = 40 ml^{-1}. Many traditional process lines have much larger (often very contaminated) dead spaces, and temperatures as well as growth rates can be higher.

59.3.2 Specific Requirements for Closed Plants

Equipment for Aseptic Processing

Bacteria Tightness. Equipment for aseptic processing must be cleanable, pasteurizable, or sterilizable (depending on product characteristics and whether or not heat-resistant spore-forming bacteria are able to grow in the product) and, in addition, downstream from the heating section of the pasteurization or sterilization plant, prevent the ingress of microorganisms (ie, be bacteria-tight).

Migration of Microorganisms through Microscopic Leaks. Microorganisms may penetrate through very small leaks. There is considerable evidence that microorganisms may pass microscopic openings very rapidly and that pressure differences may retard but not prevent passage, even if the pressure difference is as high as 0.5 bar. *Serratia marcescens* may move at a speed of 160 mm h^{-1}.[47] Motile bacteria may therefore propel themselves against the flow of liquid through a leak. This is particularly so as a layer of several micrometers of liquid along a wall is unlikely to move at all. Microorganisms, motile or not, may also grow through a passage by forming a biofilm on the surface (Figure 59–12). Data on the migration of microorganisms through microscopic passages indicate that the passage of microorganisms through holes of a few micrometers in diameter in a plate of 0.1 mm thickness:[5]

- may be virtually instantaneous if there is no pressure difference
- is hardly affected by a pressure difference of 0.05 bar
- is retarded by a pressure difference of 0.5 bar; *Serratia marcescens* may need approximately 40 hours under such conditions
- *Klebsiella terrigena* (used as nonmotile test organism) may cross within less than 20 hours, with or without pressure difference (of up to 0.5 bar).

In plate heat-exchangers, the high velocity of product between the plates may assist migration of microorganisms through leaks ("vacuum" pump effect).

The above data should be considered when deciding on safe run times, in particular if attempting to increase run times beyond those with which there is long experience. Depending on the type of hazard and the risk, it may be necessary to install a separation loop containing a microbicidal solution between the pasteurized (or sterilized) product and the cooling medium (incoming raw product or cooling water).[38]

Leaks Resulting from Corrosion. Corrosion not only affects the surface roughness of stainless steel, but may also result in leaks. This is the case when the chemically very resistant chromium dioxide sur-

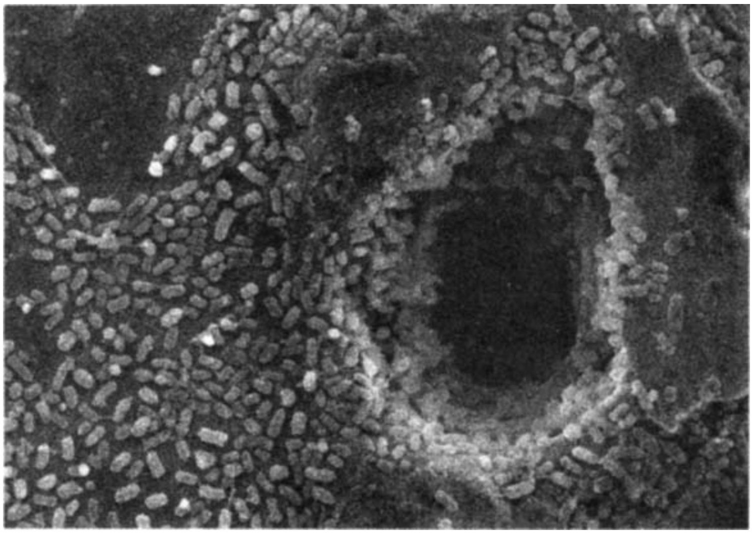

Figure 59–12 Scanning Electron Micrograph showing *Klebsiella terrigena* Growing on the Surface of a 6-µm Hole in a 0.1 mm thick Steel Disk. Courtesy of Unilever Research Vlaardingen.

face layer of stainless steel is damaged, giving chloride (and other) ions access to the iron atoms underneath. This usually results in pitting and development of leaks. Depending on the ionic composition of the product and on the chemicals used for cleaning and sanitation, it may be recommendable to choose other types of metal, such as duplex steels, titanium, or Incoloy.[17] When standing idle, such as on weekends, there should be no chloride-containing liquids left behind in equipment. Such residues are likely to dry out, gradually increasing the chloride concentration and thereby the corrosivity of the residue.

Heat-Exchanger Plates. The thinner the plates, the better the heat transfer and the more efficient the heat-exchanger. Over the past 10 years or so, to save stainless steel (and reduce the weight of the equipment), the thickness of heat-exchanger plates has been gradually decreased. Newly built units usually now have plates of 0.5 mm or even less. During pressing of the plates into the desired shape, locally the plates may be even thinner. This increases the risk of pinholes being present even in new plates. It may be important therefore, depending on the application and the position in the process line, to request that the plates supplied have been tested for the absence of pinholes.

If piston pumps are used to pass the liquid to be sterilized through a plate heat-exchanger, the pulsations generated may cause the plates to bounce with considerable force. The result is cracking of the chromium oxide surface layer. It may be necessary therefore to install pulsation dampeners (of a type that does not create a dead leg). The plates in the regeneration section of a heat-exchanger must be replaced before microscopic holes develop.

Other Heat-Exchangers. Shell and tube heat-exchangers may have the shell welded to the product pipes, in which case corrosion may result from improper welding. Cooling medium may enter the product unnoticed. In other designs, the seal between product pipes and shell is by means of O-rings. Apart from the cleaning and decontamination problems in such cases, the (many) O-rings are difficult to reach for inspection and replacement. Due to aging, but also due to movement of the O-rings in their grooves as a result of thermal expansion and pressure fluctuations, the rate of wear may be considerable and leaks may develop unnoticed.

The problems of poor welding practice apply equally to scraped/swept surface heat-exchangers (SSHE). Additionally, SSHE have shaft seals, which may be of nonhygienic design.

Leaks across Static Seals Caused by Pressure Fluctuations. If elastomer O-rings are used in the usual standard O-ring grooves, pressure differences will cause movement of the rings in their grooves. Thereby the surfaces of the rings alternatingly contact the nonproduct side and the product side of the grooves, transporting microorganisms from the outside to the inside of the equipment (Figure 59–13). Thus such constructions should not be used. Seals should be made as discussed above.

Leaks due to Differences in Thermal Expansion. As can be concluded from the paragraph on nonresilient gasket materials in the section on "Cleanability" above, sterilization of equipment with seals made from nonresilient materials, such as PTFE, is likely to cause gaps of many micrometers wide, enough for microorganisms to pass. Such materials therefore must not be used in equipment to be used for aseptic process lines. If they cannot be avoided because of the resistance to corrosion needed in a certain process step, they must be supported by a sufficiently resilient elastomer (Figure 59–14).

Migration through Dynamic Seals. Axial movement of shafts self-evidently will assist the transport of microorganisms (in a similar way as the O-rings do in case of pressure fluctuations). Not only reciprocating shafts of, for example, piston pumps and lip-seal valves, but also rotary shafts show at least some axial movement. Therefore, if the ingress of microorganisms must be prevented, reciprocating shaft seals should not be in contact with the product side. This can be achieved by separating the shaft seal from the product by means of flexible diaphragms of bellows. Also, equipment without a dynamic seal may be chosen, such as peristaltic pumps, diaphragm (membrane) pumps, and diaphragm valves. Rotary seals might be avoided by using magnetic drives. Although this may be feasible, costs and maximum available torque may be limiting.

Antimicrobial Barrier Systems. Where for whatever reason, dynamic seals cannot be avoided, such as in centrifugal pumps, stirrer assemblies, SSHE, and high-pressure pumps and homogenizers, a double-seal system should be used. The space between the seals is to be flushed with a microbicidal fluid, which may be steam, hot water, or a chemical solution. The seal at the product side should be of

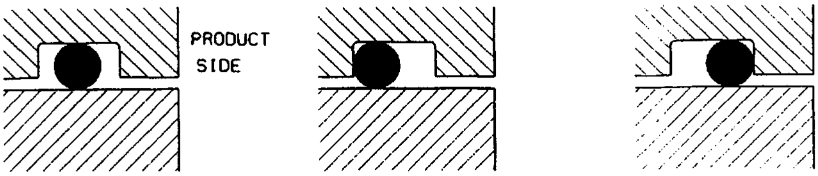

Figure 59–13 Movement of an O-ring in its Groove as a Result of Variations in Pressure. Courtesy of Unilever Research Vlaardingen.

hygienic design and therefore be a well-designed mechanical seal. Some mechanical seal manufacturers can deliver seals in which the complex, difficult-to-clean components are completely enclosed by an easily cleanable diaphragm. The seal at the nonproduct side need not be hygienic and can be of a simpler, cheap (but nevertheless reliable) design. The distance between the two seals must be larger than the axial movement of the shaft, as otherwise microorganisms may be conveyed from the outside to the product side in too short a time for inactivation. Figure 59–15 shows the principle of such an antimicrobial barrier system.

To prevent too fast fouling, a chemical solution is often preferred to steam or hot water. Care should be taken that the chemical chosen is effective and remains effective for the duration of the process run or runs intended. Many antimicrobial chemicals lose their activity in the presence of (too much) organic material, in particular, proteins. Suitable solutions include diluted hydrogen peroxide (>3%), sorbic acid (0.2-0.5%; pH <5.2), and glutaraldehyde (2%; pH8). Leakage of product may cause a change in pH of the solution and the pH often is important for the effect. If too much product has passed the seal closest to the product side, the solution meant to kill microorganisms may actually become a microbial growth medium. Therefore, frequent checking and timely replacement of any antimicrobial barrier liquid are important and should be part of the operating instructions. If a chemical solution is used to prevent contamination of the product processed, the space between the seals must be at atmospheric pressure, which can be achieved easily by ensuring that the return line of the circuit ends in the storage vessel without obstacles. As the product may also be at atmospheric pressure, there is still a risk, although small, of leakage of antimicrobial liquid to the product; therefore, only chemicals that cannot make the product toxic must be used. To be able to detect a leak, the level of the liquid in the storage vessel should be monitored. If leakage occurs, the result will be a very diluted concentration in the product. Assuming a product flow of 1000 kg h^{-1} of product (which is low), passing a seal, which leaks 10 g h^{-1} (which is high), and if the concentration of antimicrobial chemical is 3% (w/w), the concentration in the final product will be 0.3 mg kg^{-1}, which is far below the acceptable concentration of the chemicals discussed.

Mixproof Valves. Normal mixproof valves have two seats, while the space between the seals is open to the environment (Figure 59–16). They have been

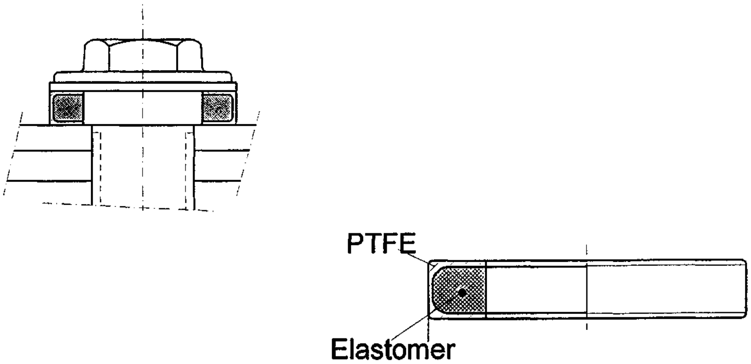

Figure 59–14 Chemically Resistant Gasket with Elastomeric Inlay To Provide Resilience (PTFE = polytetrafluoroethylene). Courtesy of Unilever Research Vlaardingen.

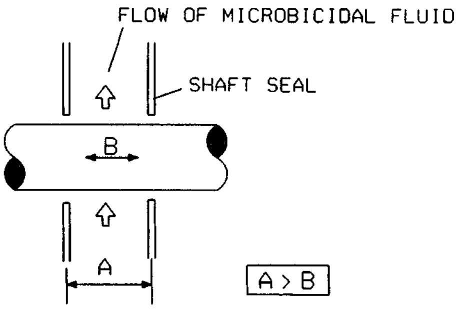

Figure 59–15 Schematic Presentation of a Double-Shaft Seal. The distance between the seals (A) must be larger than the stroke (B) of the shaft. Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

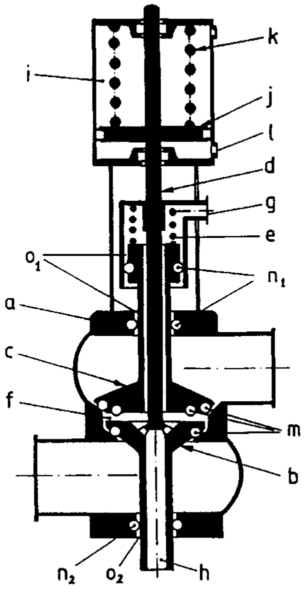

Figure 59–16 Schematic Presentation of a Double-sealed Valve with Leakage Indicators. (A), housing, (B), lower valve head; (C), upper valve head; (D), main valve rod; (E), locking spring for the valve head; (F), leakage space; (G), joint for cleaning the leakage space; (H), exhaust for leakage; (I), pneumatic pressure drive; (J), lifting piston; (K), main locking spring; (L), pressure-air connection; (M), sealing rings of the valve heads; (N_1, N_2) packings of the valve rod; (O_1, O_2,) seating for the valve rod. *Source:* Copyright © 1989, G. Hauser, R. Mitchell, and K. Sommer.

designed to drain any leakage from the seats. That way such leakages cannot build up pressure so that chemicals used for cleaning do not pass the seat at the product side of the valve. The construction of such valves is rather complex, and many types cannot be drained completely. Water, or worse, product residues, may be sitting on the product side seal for a long time and allow the multiplication of microorganisms. Any microscopic leak of that seal may therefore result in microbial contamination of the product. In aseptic process lines, mixproof valves should be used only if the space between the two seats can be flushed continually with a suitable microbicidal fluid. To avoid the risk of contamination with microorganisms, mixproof valves should not be used to separate raw and decontaminated product unless the antimicrobial fluid between the seats is controlled and monitored continually, to give an alarm in case of failure. A detailed discussion of various types of mixproof valves occurs in the literature.[29]

Sterility Barriers. Figure 59–11 shows two layouts of valves and associated pipework for the transfer of product under sterile conditions to an aseptic tank and to protect the product in the tank from contamination while a product line is unsterile. Figure 59–11A shows a typical sterile barrier. When the plant and tank are sterile and product is to be transferred to the tank, valves 3 and 4 are closed and 1 and 2 opened. If the process plant becomes unsterile and hence it is necessary to protect the product in the tank, valves 1 and 2 are closed and steam purges the short section of pipe between them. A bypass valve also must be fitted such that the back of valve 4 can be steamed to prevent any potential contamination via valve 4. This also prevents condensate accumulating in the steam supply line. A temperature sensor in the outlet line, between valve 4 and the orifice/steam trap, enables the status of the barrier to be monitored and ensures that the barrier temperature is always sufficient. In some cases, an antimicrobial fluid may be used in place of steam within the barrier. Note the potential dead areas. Figure 59–11B shows a similar sterile barrier, but one that is free from dead legs, using an aseptic double seat valve. The design of the valve must be such that the distance between the seals is longer than the stroke of the shaft.[30]

Testing Bacteria-Tightness of Process Equipment. The bacteria-tightness of equipment can be tested by a standardized method,[50] which often is performed in conjunction with the sterilization test described earlier. In this test, equipment is sterilized and filled with a sterile liquid culture medium. The outside of all suspected areas of the equipment to be tested are wetted with a culture of *Serratia marcescens*, a very motile bacterium. This is repeated several times, while any movable component is actuated. If the medium in the equipment remains sterile, it is apparently bacteria-tight.

59.3.3 Decontamination of Air

Airborne Microorganisms

In most environments the concentration of microorganisms in the air ranges between 100 and 1000 m^{-3}; in agricultural areas, the concentration may be much higher. In coastal areas, the concentration often is low. Airborne microorganisms may be single spores, such as conidiospores of molds like *Penicillium* and *Aspergillus*. Bacterial spores, however, often occur as clusters, surrounded by a mixture of inorganic and organic material, remnants of the substrate in which they have developed. High concentrations of vegetative bacteria usually are found close to their origin only, such as near wastewater treatment plants, although some vegetative bacteria, such as some micrococci, are tough enough to survive in air for a long time. Their origin can also be from humans and from raw materials and products during processing. In many instances, airborne microorganisms are associated with dust particles. A recent study[35] showed the aerodynamic diameter of airborne particles containing microorganisms averaged approximately 9 µm.

Decontamination by Incineration

Air can be sterilized within less than a second by heating to a temperature of 350° C.[23] At such a temperature, organic materials are destroyed together with all known microorganisms. Incinerators, therefore, are probably more reliable than filters, provided that their design guarantees that all air is heated to the required temperature; therefore, an incinerator must be equipped with a reliable temperature control system. Although this method does not require much energy because of the low heat capacity of air, for most applications the method is more expensive than filtration.

Decontamination of Air by Filtration

Filtration is based on various mechanisms of particle retention. Depth filters usually rely on entrapment of particles in a labyrinth of fibers and electrostatic attraction. Membrane filters rely on pore sizes. Membrane filters have a much higher resistance than depth filters and are therefore not normally used in the food industry. Industrial high efficiency particulate air (HEPA) filters are made from corrugated sheets of compact fibrous material that is mounted in cartridges using a glue or resin. The cartridges in turn fit in the filter houses that are mounted in the air ducts (air for clean rooms) or pipelines (process air). As a leak may virtually annihilate the effect of the filter (if 1% of air is passing between housing and cartridge, the reduction in concentration of microorganisms will be less than 100-fold), the installation of the cartridges must be done very carefully, with particular attention to the gaskets used to seal between cartridge and housing. For critical applications it may be advisable to use two filter assemblies in series and/or to have the filter assemblies tested before installation and use.[34] The literature discusses air treatment of clean rooms for food processing, covering such aspects as choice of filters, filtration efficiency, and the testing and maintenance of filters.[3,7,37] Chapter 15 of Wang[55] "Air Filtration, Air-Handling Units and Packaged Units" also provides considerable useful information on filtration in air conditioning and refrigeration installations.

59.3.4 Packaging

If a product has been produced and has been pasteurized or sterilized correctly, it must be ascertained that the next step, packaging, does not adversely affect the condition of that product; therefore, care must be taken that no unacceptable recontamination takes place during the transfer of the product to the packaging equipment and during the packaging operation itself. If the process line and the packing machine are cleaned and decontaminated independently, which usually is the case, the connection between the two must not impair the hygienic condition achieved. In case of aseptic packing, making the connection must also be done in an aseptic way. This requires that the connection made is thoroughly decontaminated before starting the flow of product. Equally, during cleaning and decontamination of the packing machine the process line or aseptic storage tank must be protected against contamination by the cleaning chemicals and *vice versa*.

The Influence of the Environment of the Packing Machine

The risk of contamination of a surface with microorganisms will always be proportional to the concentration of microorganisms in the immediate environment and the time of exposure. Therefore, to reduce the risk of infection during packaging, it may help to reduce the concentration of microorganisms in the environment and to keep the time of exposure to the environment of the product side of the packing material and the product to a minimum. The sedimentation velocity of microorganisms present in the air is in the order of $v_s = 0.003\ m\ s^{-1}$.[35] A simple calculation shows that the infection of a product by microorganisms in the air can indeed be significant: if the concentration in the air is $x_{air} = 1000\ m^{-3}$, the time of exposure is $t = 5$ seconds and the exposed surface area is $A = 0.01\ m^2$ (ie, 100 x 100 mm), the number of microorganisms settling on that surface will be:

$$n = v_s \cdot x_{air} \cdot t \cdot A = 0.15$$

In words, under the conditions assumed in this example 15% of the surfaces exposed will be infected with one microorganism. This may or may not be acceptable in case of hygienic packing; it certainly will be unacceptable for aseptic packaging. The risk of contamination may be reduced by simple measures, such as the installation of a tunnel over the area where the packing material and the products are exposed. If this would not be effective enough, sterile air may be supplied to the tunnel.

The Influence of the Microbiological Quality of the Packing Material

The packing material may contain more microorganisms than desirable. If so, improvement of the quality may be negotiated with the manufacturer. A better quality may be obtained following a number of measures to be taken by the manufacturer, such as a cleaner environment in the factory, more hygienic handling of the material, removal of static electricity, better protection during transport, and avoiding the use of heavily contaminated cartons for packing of the packaging material. It may be more secure to decontaminate the packing material on the packing machine just before filling. This may be done using hydrogen peroxide, heat, UV light, or other treatments, either individually or in combination. If acid products or products with a sufficiently low water activity are packed, the use of moist heat can be attractive as it seems to be effective against vegetative microorganisms without using chemicals.[46]

To prevent contamination of packing material, the following precautions may be needed:[43]

- protecting (by the packing material manufacturer) the packing material
- cleaning of the outer material before opening
- removing dust from the air
- avoidance of manual handling
- keeping the packing material storage area clean and dry
- removing static electric charges.

Hygienic Filling and Packaging

When packing microbially unstable products, which are not to be in-pack sterilized or pasteurized, it is essential that the residence time of the product in the packing machine and the temperatures in the machine are controlled so that microbial growth does not occur. This is particularly important during production stops. If the duration of stops exceeds a certain time, the packing machine may have to be cleaned and decontaminated before restarting. When cleaning filling and packaging equipment used for dry products, wet cleaning should be avoided; dry cleaning methods are discussed in Chapter 58.

Condensate formed during filling of relatively hot products or formed on filling nozzles during filling of products that are at a low temperature relative to the ambient temperature should be prevented from contaminating the product. Where possible, moving parts of fillers should be cleaned outside the production area. The build-up of product around the filling nozzle is impossible to avoid, and is a potential source of contamination of the product flow; such build-up may determine the run time of the filling equipment before cleaning is necessary.

Aseptic filling and packaging requires the maintenance of commercial sterility of the product during filling and packaging. The design and operation of aseptic packer is considered in Cerf and Brissenden[9] and the control of sealing of packs in Mostert et al.[42]

59.3.5 Examples of Equipment of Wrong and Correct Design

General

Typical examples of designs that may easily result in microbiological problems are given in Figures 59–17 through 59–21. The use of bolts should be avoided, but where unavoidable, at least the crevices they create should be sealed (Figure 59–17). Sharp corners should be avoided (Figure 59–18); exceptions are constructions where the sharp corner is continually swept such as in lobe pumps. Welds must be smooth when made and remain smooth in use. This requires skilled welders doing a good job. Figure 59–19 presents schematically welds easily causing hygiene risks as well as correct welds for the same purpose. Figure 59–20 shows the results of improper welding, such as misalignment, lack of penetration, and lack of gas shielding. Figure 59–21 shows the results of correct welding.

Open Plant

Figure 59–22 shows how drainable equipment should be designed and installed. Figure 59–23

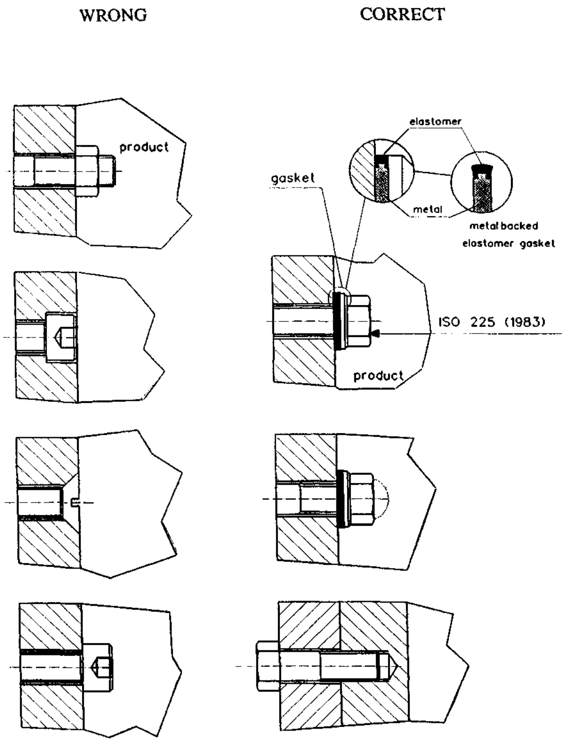

Figure 59–17 Examples of Good and Bad Designs of Connections. Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

shows the incorrect and correct ways of installing fixed equipment so that it can be cleaned:

- Diagram A (left) shows incorrect fixing of equipment to the floor with insufficient clearance, non-radiused, improperly fixed feet, sharp corners, and crevices at the fixing spot causing hygiene risks
- Diagram A (right) shows feet properly fixed to rounded pedestals or sealed to the floor with sufficient clearance to allow effective cleaning

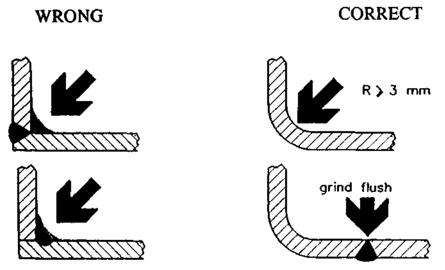

Figure 59–18 Examples of Good and Bad Corners. Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

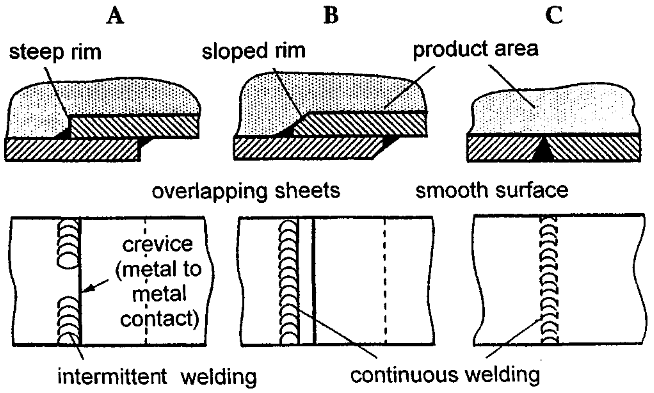

Figure 59–19 Examples of Welded Joints. (**A**), Poor design: overlapped sheets with intermittently welded seams create crevices and metal-to-metal contact areas between seams. (**B**), An improved design: overlapped sheet having continuous welds and sloped rims for easy cleaning. (**C**), Best design: smooth continuously welded sheets. Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

- Diagram B (left) shows how equipment incorrectly attached to a wall can accumulate dirt and would be difficult to clean
- Diagram B (right) shows the use of radiused horizontal supports and a sufficient gap for cleaning between the wall and the equipment or direct fixing to the wall and sealing any gap with a sealing compound
- Diagram C (left) shows poor location of equipment under a tank, making maintenance of the equipment and cleaning under the tank difficult
- Diagram C (right) shows better positioning of the equipment
- Diagram D (left) shows a raised and open walkway over equipment and the potential for contamination from personnel using the walkway
- Diagram D (right) shows a better, enclosed design.

Figure 59–24 shows various hygienic and unhygienic designs of conveyor belt. It is very important that conveyor belting does not absorb moisture as this will lead to microbial growth and contamination of product carried on the conveyor. Once microorganisms have colonized the inner fabric of the belt, it is impossible to kill them. Damaged belts must always be replaced and their edges sealed. Even when conveyors are properly designed and maintained, cleaning may still be difficult; thorough

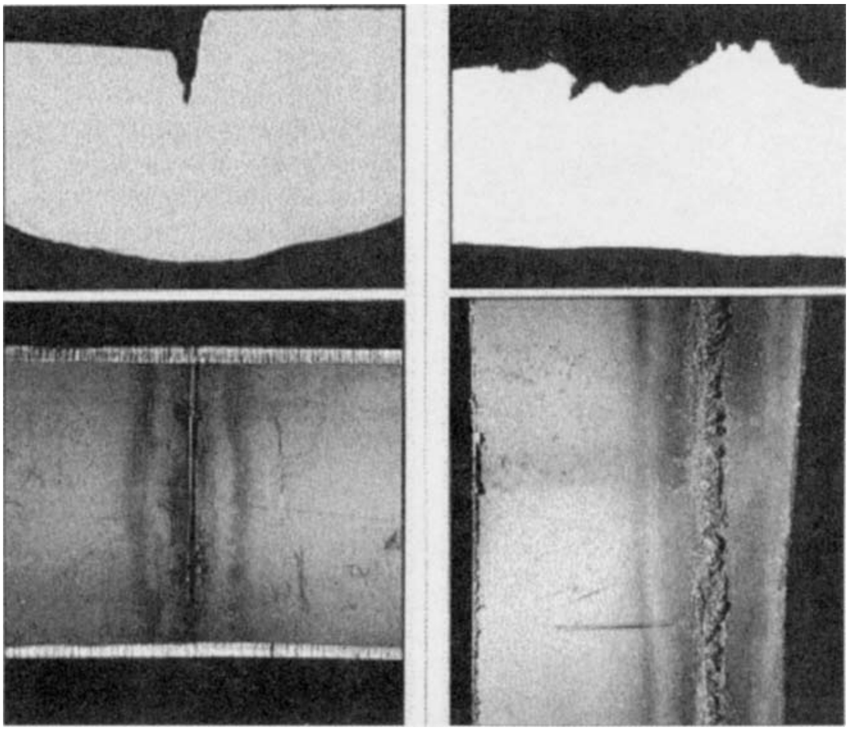

Figure 59–20 Photograph of Cross Section of a Weld Made without Adequate Gas Shielding (right) and of a Weld, between Improperly Aligned Pipe Ends, Also Lacking Penetration (left). *Source:* Reprinted with permission from C.A. Eastwood, D.L. Woodall, D.A. Timperley et al., 1993, Welding Stainless Steel To Meet Hygienic Requirements. *EHEDG Document 9*, as summarized in *Trends in Food Science Technology*, Vol. 4, pp. 306–310, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

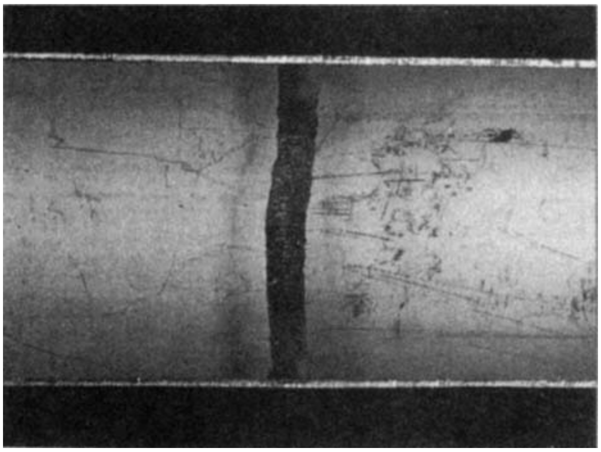

Figure 59–21 Typical Hand-made High-quality Weld. *Source:* Reprinted with permission from C.A. Eastwood, D.L. Woodall, D.A. Timperley et al., 1993, Welding Stainless Steel to Meet Hygienic Requirements. *EHEDG Document 9*, as summarized in *Trends in Food Science Technology*, Vol. 4, pp. 306–310, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

cleaning and inspection of cleanliness after cleaning are essential.

Curiel discusses many details of the hygienic design of open plant.[19]

Closed Plant

Figure 59–25 shows how a lid of a tank, intended to protect the product, may accumulate dirt that will enter the product in the tank when the lid is opened, next to a correct design. Policy should be that tanks are not opened at all during production unless absolutely necessary. Figures 59–26, 59–27, and 59–28 present ways to ensure that closed equipment can be drained.

A common mistake in the construction of bypasses for positive displacement pumps, causing a large dead area filled with product, is shown in Figure 59–29. With almost the same components, dead areas can be avoided completely. Figure 59–30 shows wrong and correct designs of couplings, with attention to (mis)alignment and the correct use of gaskets. Flexible hoses frequently are used to connect mov-

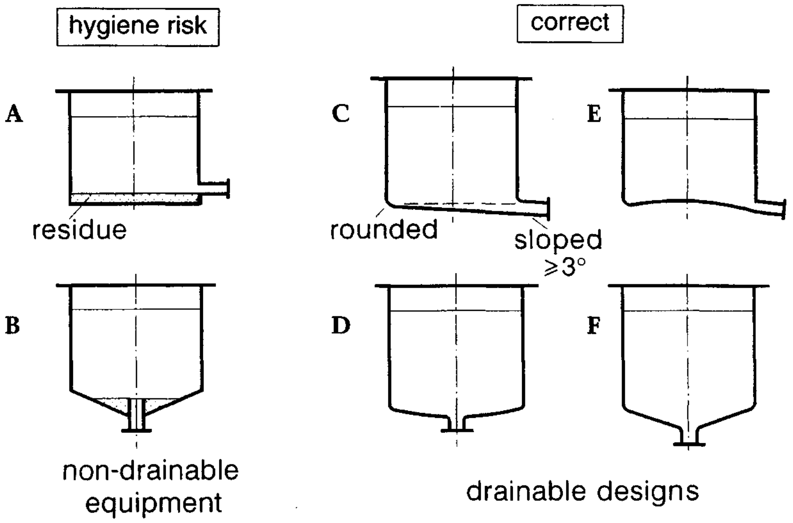

Figure 59–22 Drainability of Equipment. In **A** and **B** discharge outlets above the lowest level of the equipment prevent self-draining. Diagrams **C–F** demonstrate self-drainable designs with discharge openings at the lowest level, sloped bottoms (≥ 3°), and well-rounded corners. Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

ing and static parts of process lines (eg, movable dosing heads on filling machines). Figure 59–31 shows the noncleanable crevice created by the traditional way of mounting hoses to pipes and how this can be done correctly (ie, without crevices). It is strongly recommended that the EHEDG guidelines on closed plant equipment are followed.[18]

59.4 DESIGN OF PROCESS LINES

59.4.1 General

The choice of equipment used to build a process line should be based on a hazard assessment, which takes into account the microbial susceptibility of the product, the stage in the process and the required cleaning frequency, and the conditions of storage and the shelf life of the product. For handling raw materials, straight from the field, the requirements will be different from those for washed or cooked product. The aim should be that none of the process steps decreases the microbiological quality of the product. This also requires measures to prevent cross contamination (see also Section 59.2.1). Having selected the appropriate pieces of equipment for a process line, it is extremely important to ensure that the design of the whole line allows good cleanability and sanitizability (disinfection, pasteurization, sterilization) and for aseptic processes bacteria-tightness requirements are retained.

Cleaning

It is essential that the cleaning program ensures that all parts of the process line are cleaned, and that all components and all product contact surfaces are also appropriately cleaned. In practice, this may mean that equipment has to be dismantled for access by brushes or water lances. In closed process lines, the cleaning fluids may have to be circulated several times and, during cleaning, movable parts (such as shafts of valves, stirrers, scraped surface heat exchangers, and pumps) need to be actuated a number of times or for a certain period of time. In particular, valves must be open to be able to clean their seats, and stirrer shafts must rotate to have soil removed from within, for example, the bottom slide bearings. These actions will increase the total cleaning time, and it is tempting to reduce the times for the various steps. As soil residues effectively hamper inactivation of microorganisms, doing so may have detrimental consequences.

In the design of a closed cleaning system, variations in dimensions in the pipework must be taken into account. The capacity of the cleaning pump(s) must be chosen to cope with the largest cross section to be cleaned in order that the velocity of the clean-

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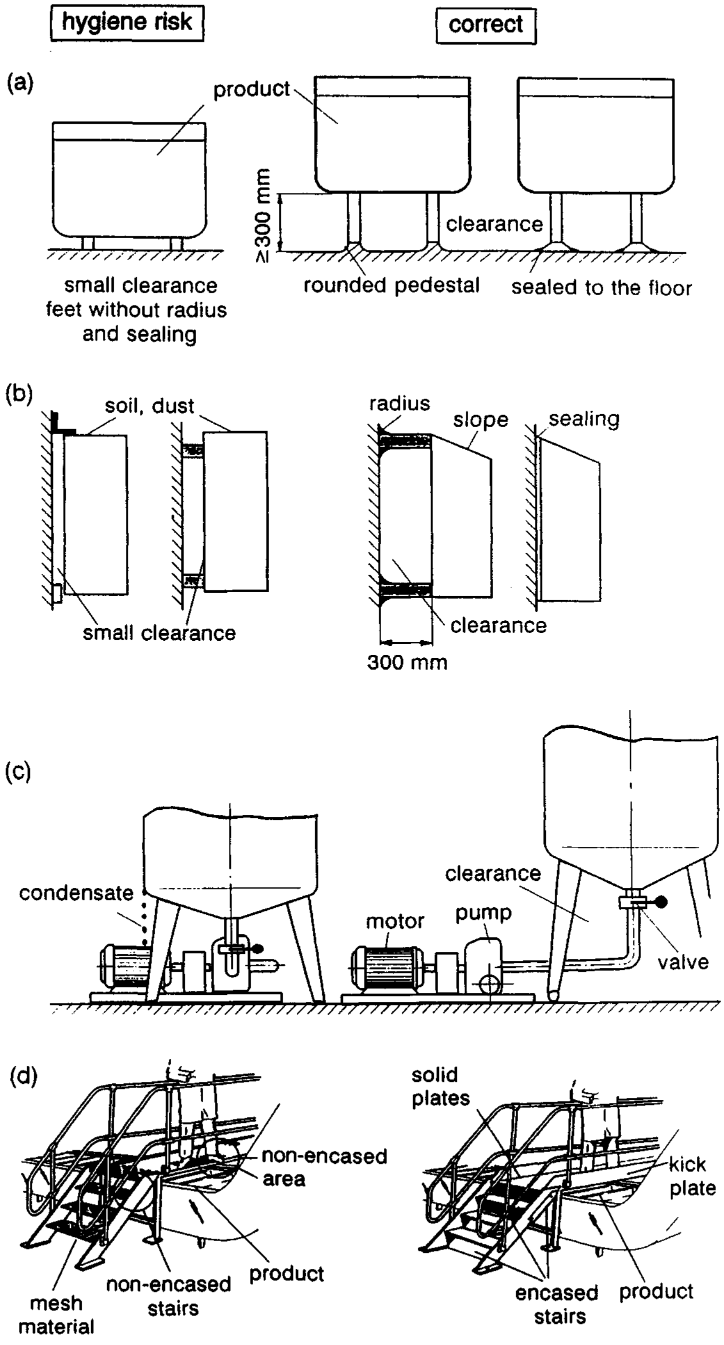

Figure 59–23 Examples of Incorrect and Correct Installation of Equipment. *Source:* Reprinted with permission from G.J. Curiel, G. Hauser, D.A. Temperley, 1995, Hygienic Design of Equipment for Open Processing, *EHEDG Document 13*, as summarized in *Trends in Food Science Technology*, Vol. 13, pp. 305–310, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

ing fluid is not lower than that intended in any part of the process line. Effective cleaning requires a velocity of cleaning liquid of 1.5 m s^{-1}. Lower velocities may dramatically increase the time required for cleaning.[48]

Gould[28] presents a very useful overview of cleaning and sanitation of both open and closed plants. It is strongly recommended to design a cleaning system in consultation with a specialist cleaning company.

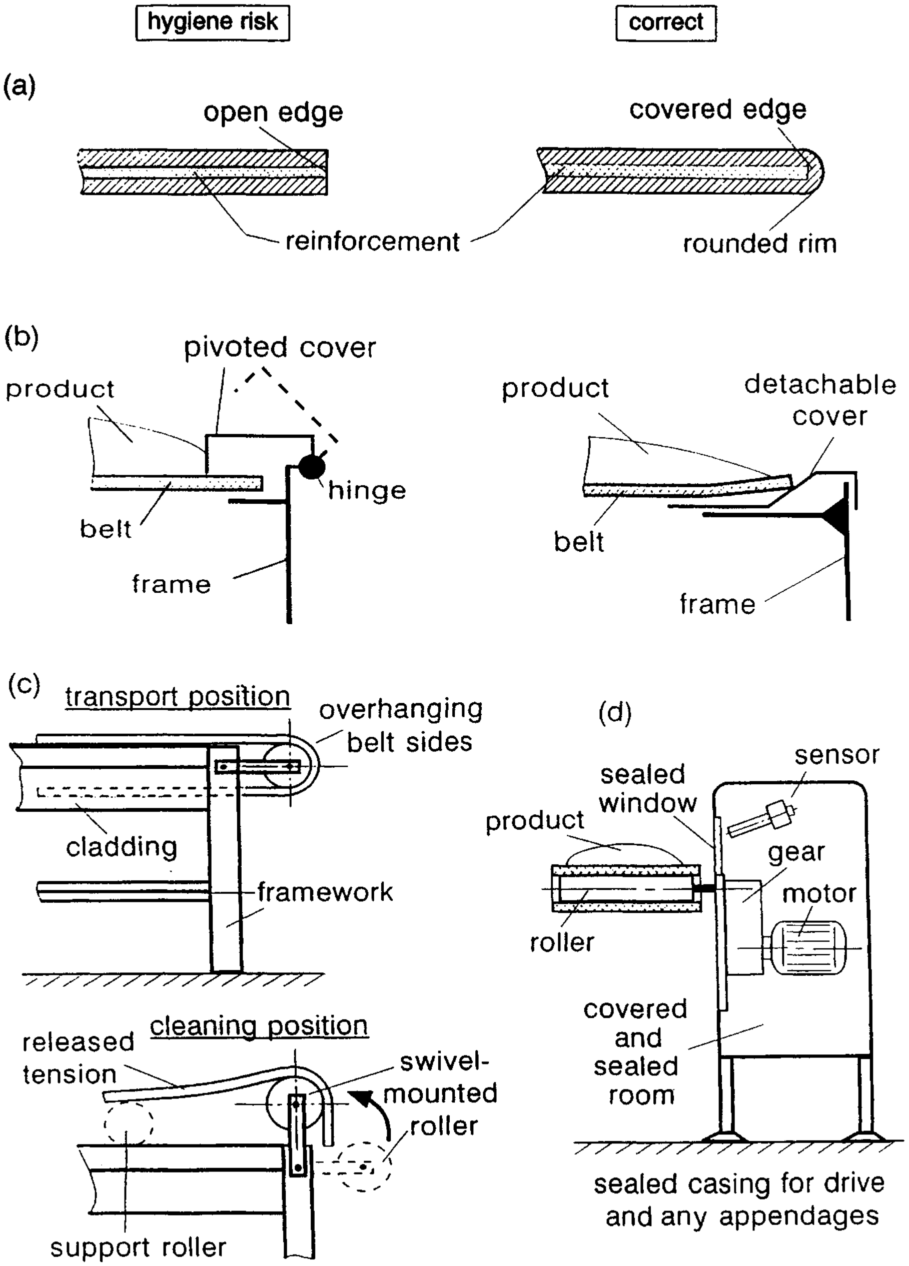

Figure 59–24 Examples of Incorrectly and Correctly Designed Conveyor Belts. *Source:* Reprinted with permission from G.J. Curiel, G. Hauser, D.A. Temperley, 1995, Hygienic Design of Equipment for Open Processing, *EHEDG Document 13*, as summarized in *Trends in Food Science Technology*, Vol. 13, pp. 305–310, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

Decontamination

A clean process line can be readily decontaminated, provided that all product contact surfaces receive the correct treatment. If heat is used, not only the temperature needs to be taken into account, but also the available water (a_w). Thus if steam is used, it must be assured that the steam is saturated. It must also be known how long it takes for the coldest spot in the process line to reach the required temperature. As discussed under thermal decontamination of process equipment, even if clean, surfaces may remain dry if protected by elastomers; hence the decontamination procedure will be ineffective. This will occur

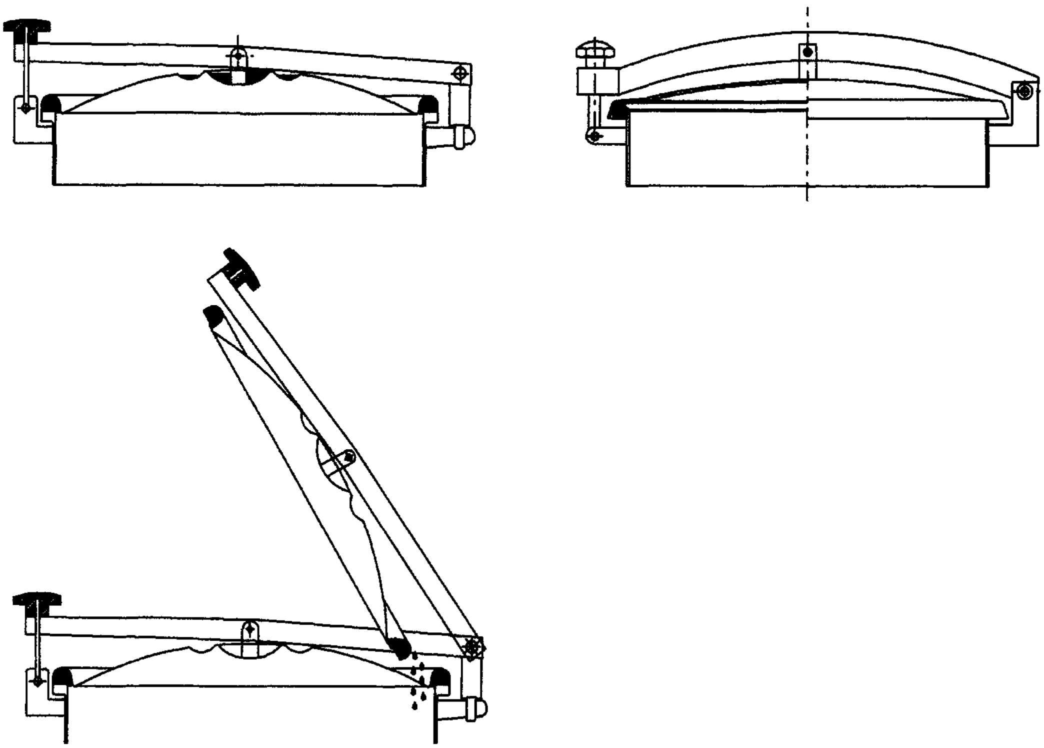

Figure 59–25 Examples of Tank Lids (Manhole Hole Cover). The lid on the left is a poor design and dust, etc., accumulating on the cover will fall into the tank when the cover is lifted. The manhole cover on the right is a better design. Courtesy of Unilever Research Vlaardingen.

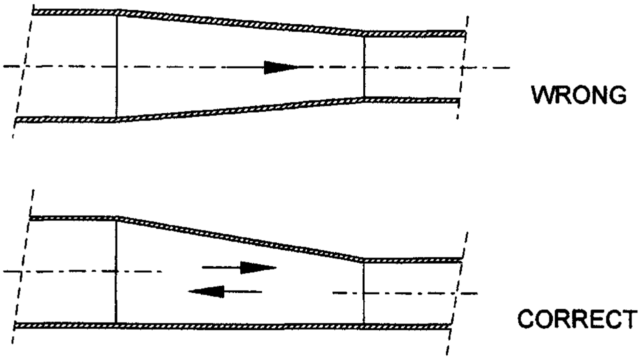

Figure 59–26 Use of Concentric and Eccentric Reducers. Concentric reducers in horizontal pipelines may hamper draining, depending on the direction of the flow. An eccentric reducer, properly installed, will not affect draining, independent on the direction of the flow. *Source:* Reprinted with permission from G.J. Curiel, G. Hauser, P. Peschel, et al., Hygienic Design of Closed Equipment for the Processing of Liquid Food. *EHEDG Document 10*, as summarized in *Trends in Food Science Technology*, Vol. 4, pp. 375–379, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

if valves are closed. Thus, valves must be opened long enough to ensure that the seat is properly freed from relevant microorganisms; as with cleaning, this may require circulation of the heating medium through the open valve.

Dead ends in pipelines may hamper decontamination by heat or chemicals. If hot water is used to pasteurize a process line, an upward-pointing leg of a T-piece will trap air, thereby reducing the rate of heat transfer and causing the decontamination of the dead leg and what is connected to it to fail. If a process line is sterilized with steam, an upward-pointing dead leg, if clean and not too long, will probably be decontaminated properly, as steam will condense on the surfaces of the leg and fall down to let more steam condense and give off its energy. With steam sterilization, downward-pointing legs give problems. Here condensation will be collected in the leg and hamper heat transfer: the temperature thus will be too low for complete sterilization (unless the presence of the leg has been taken into account in defining sterilization procedures). A properly designed

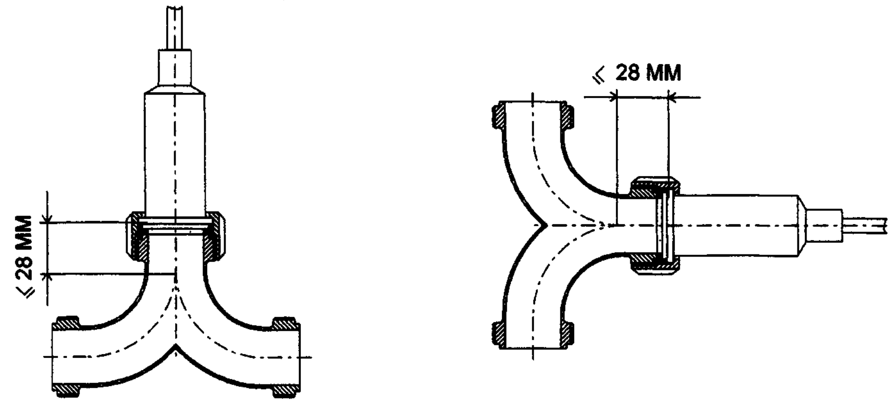

Figure 59–27 Use of Swept Tees. These avoid dead areas and thus facilitate cleaning but if mounted in a horizontal pipeline, they hamper drainage. Swept tees must be used in vertical pipelines only. *Source:* Reprinted with permission from G.J. Curiel, G. Hauser, P. Peschel, et al, Hygienic Design of Closed Equipment for the Processing of Liquid Food. *EHEDG Document 10*, as summarized in *Trends in Food Science Technology*, Vol. 4, pp. 375–379, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

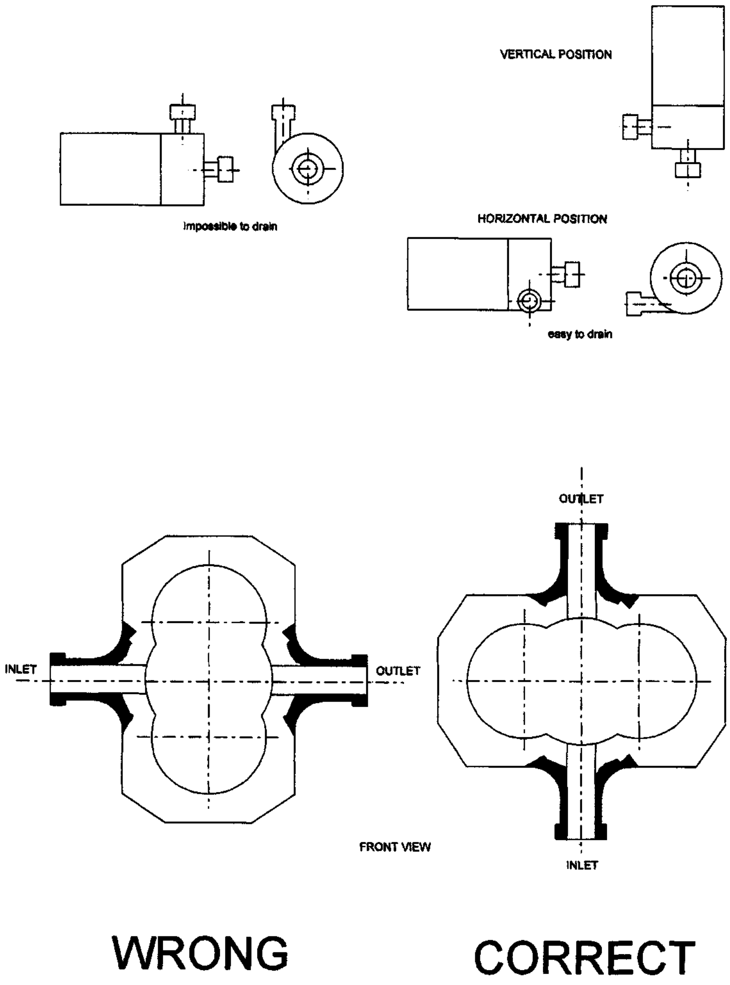

Figure 59–28 Positioning of Centrifugal and Lobe Pumps. It is important to position centrifugal (top) and lobe (bottom) pumps in such a way that they are drainable (right). Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

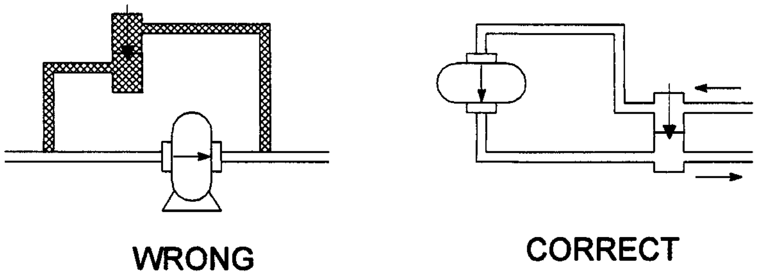

Figure 59–29 Location of Relief/Bypass Valve for a Positive Displacement Pump. The pressure relief/bypass valve for the positive displacement pump shown at left has a dead leg with a large amount of product (shaded area) when the valve is closed, as shown (note also that the pump is not in a position that allows draining). An almost identical valve, mounted as shown in the arrangement shown at right, avoids dead legs in the bypass and allows draining of the pump. *Source:* Reprinted with permission from G.J. Curiel, G. Hauser, P. Peschel, et al, Hygienic Design of Closed Equipment for the Processing of Liquid Food. *EHEDG Document 10,* as summarized in *Trends in Food Science Technology,* Vol. 4, pp. 375–379, Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

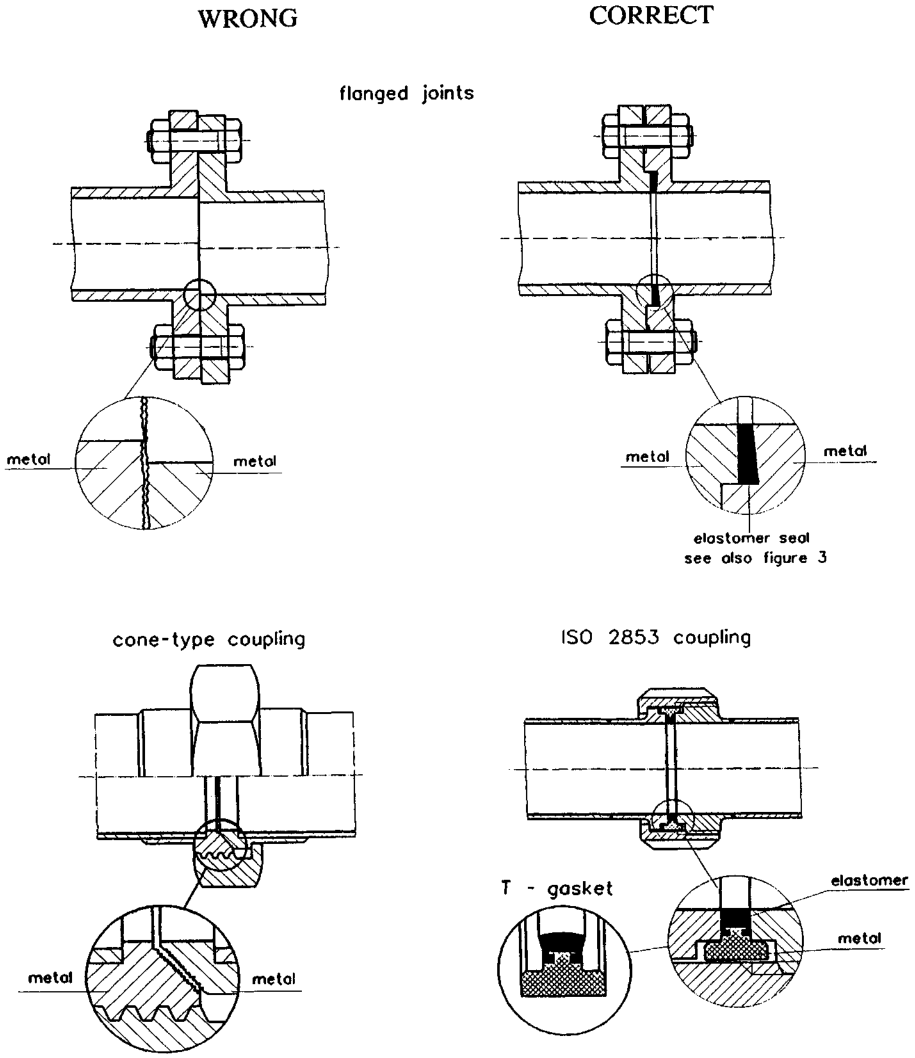

Figure 59–30 Design of Pipe Coupling. The pipe couplings on the left seal between two metal surfaces and therefore have crevices, which cannot be cleaned and are unlikely to remain bacteria tight. The couplings shown on the right provide a permanent seal and are free from crevices. Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

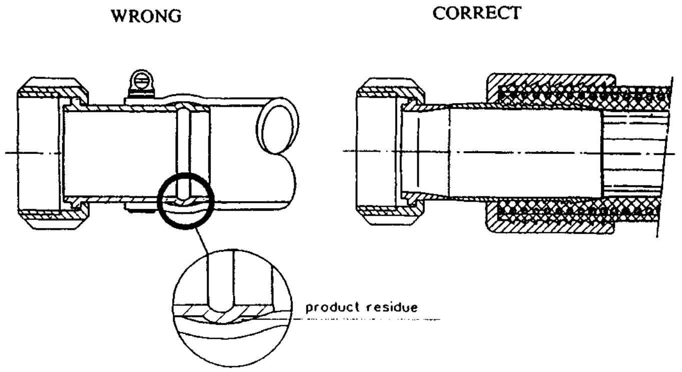

Figure 59–31 Clamping of Flexible Connections. These must be clamped in such a way that no product will be trapped between the hose and liner. Courtesy of European Hygienic Equipment Design Group (EHEDG), Brussels.

food processing line therefore should not have unnecessary dead legs. Those that are unavoidable should be in the correct position for the selected decontamination treatment.

Drainability

It must be ascertained that the self-draining characteristics of the equipment used to build the process line are retained. Often equipment can be drained only in certain positions (Figures 59–26 and 59–27) and this must be taken into account in designing process lines. Also the way the equipment is interconnected must not hamper drainage. The use of isometric three-dimensional drawings can be of great help as they will make it easy to see where draining will be hampered, which often is very difficult to see with two-dimensional drawings.

Tables, conveyor belts, and pipelines must be supported to the extent that pooling of water is prevented. If it is not possible to make a large surface or a long pipeline self-draining, extra drain points must be created. Operators must be instructed on how to use such drain points, particularly if it is necessary to dismantle or loosen components (such as pipe couplings) to allow drainage, requiring tightening or reassembling prior to resuming production.

59.4.2 Specific Requirements for Closed Processes

General

The equipment used in process lines not intended for sterilized products must at least be pasteurizable; chemical disinfection is not usually used for closed plant. Downstream from the heating section of any sterilization plant, hygiene requirements may differ depending on the stability and shelf life of the product. If the sterilized product must have a long shelf life at ambient temperature, all equipment must be sterilizable. However, if the sterilized product is intended for a limited chilled shelf life, it may be sufficient if the equipment is pasteurizable. Long shelf-life products, whether made microbially stable by sterilization (low-acid products of high water activity) or by pasteurization (acid products or products with a sufficiently low water activity), must be protected against recontamination after the heat treatment and thus require equipment that is bacteria-tight. Requirements for the production of pasteurized foods will be found in the literature.[38]

Bacteria-Tightness

Care must be taken that the connection of a dedicated cleaning system to a line intended for aseptic processing does not break the bacteria-tightness of the line. Unless the cleaning system is physically separated from the process line after cleaning, mixproof valves must be used between the cleaning system and the process line (see Section 59.3.2). To prevent the accumulation of dirt and the growth of microorganisms on the nonproduct side of the valve seat closest to the product, the space between the two seats of the valve must be flushed with an antimicrobial fluid. If the product in the process line is not too heat-sensitive, steam or hot water may be used for this purpose. Otherwise, a safe antimicrobial chemical should be used.

59.5 LEGISLATION

The European legislation on food hygiene[15,16] is firm and clear with respect to its objectives; the same applies to legislation in the US.[24–26] Such legislation does not give the details on how to comply but sets down what objectives should be achieved. The initiatives and responsibilities for achieving these standards are left with the manufacturers of food as well as with the suppliers of the machinery used to produce the food. To assist the industry to develop and design hygienic processing lines and equipment, the 3-A organization was founded in the US in 1927. In this organization, equipment manufacturers, food processors, and food inspectors work together to define the requirements for the safe manufacture of food products. Many standards and recommendations have been published. In 1989 the European Commission mandated the European federation of standardization organizations (CEN) in Brussels to produce standards on the hygienic aspects of food processing machinery. Since then, much work has been done by CEN's technical committee 153 and its many working groups and many standards have been prepared. The EHEDG[2] also has published a whole series of publications describing how to comply with hygienic requirements for the safe production of food. In addition, the EHEDG has published "standard" test methods, which can be used to verify whether equipment complies with requirements for hygienic or aseptic operation of food plants; 10 independent institutes in Europe will test equipment according to the EHEDG recommendations.[10] The 3-A and the EHEDG have worked together since 1992, with the objective to harmonize principles and requirements between Europe and North America. The International Dairy Federation also is active in the area of hygienic design of dairy plants.[9]

59.6 CONCLUDING REMARKS

Although in the past 20 years or so much food processing equipment has been designed incorporating sound hygienic design features and many factories have been built with a good understanding of their hygienic design needs, there are still many examples of new designs where there is clearly lack of understanding of the basic principles of hygienic design. To a large extent this is due to lack of awareness. Many individuals with responsibilities for the handling and processing of food have little understanding of basic hygiene measures. This results in many hundreds of thousands of food poisoning incidents per year. This ignorance applies to the entire food chain, from the farmers who grow the food, to the processors and manufacturers who make the food products, to the retailers and caterers who deliver the final product to the consumer and, indeed, includes the consumer, who handles the food in the home. There is a need for those involved in the food chain to receive better education and training in food hygiene and for better cooperation among those involved in the design of new processes and food processing equipment and premises and those that are responsible for managing food operations. The globalization of food supplies, extension of food chains, and the trend toward more convenience, less processing, and fresher tasting products will require the application of even better hygienic designs and practices.

Developments in electronics will result in sensors becoming available that are able to measure minute changes in physical parameters, including temperature, pressure, light, sound, and ultrasound. These developments are expected to play an increasingly important role in the control of food processes. Appropriate research and development will lead to devices that will warn when fouling starts or approaches the acceptable limit, when cleaning of critical surfaces is completed, when filters leak or become too resistant, when deviations from the expected flow and direction of air occur, and when the concentration of particles in the air is increasing. Gradually, such devices will tell the plant operator when to initiate which actions to ensure maximum product safety and quality. Robots will also take over many of the roles of the food operative, being able to operate under a much wider range of inimical conditions than humans.

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60

Sampling for Microbiological Analysis

Basil Jarvis

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Appendix 60–A Some terms used in statistical analysis and sampling

60.1 INTRODUCTION

The accuracy and reproducibility of results obtained from any analysis—physical, chemical, or biological—will be dependent on a number of factors associated with the methodology, the equipment, the analyst, and the extent to which the sample analyzed is truly representative of the material under examination. To obtain a reliable estimate of the characteristics of the material under test, it is essential to analyze a number of replicate samples. This is of particular importance when materials of biological origin are analyzed for microorganisms that may not be distributed randomly throughout the product. Hence, the statistics of microbial distribution introduce an overlay onto the already complex statistics of sampling and analysis. The statistical terms used in this chapter are defined in Appendix 60–A.

60.1.1 Microbial Distributions in Foods

A bacterial cell occupies a volume of approximately 1.2 μm^3; hence in a sample of 1cc of a well-mixed liquid, the relative volume occupied by 10^6 bacteria would amount to only approximately 0.0001% of that sample. In such a sample, it would therefore be reasonable to assume that the bacteria would occur randomly; that is to say, that the distribution of bacterial cells would be expected to follow a Poisson distribution. Since bacteria replicate by cell division, it would also be realistic to expect that many cells will occur as small clumps or associations, each of a variable number of cells. Hence a sample of a liquid, such as milk, might contain both randomly distributed individual cells and randomly distributed clumps of variable numbers of cells. Traditional microbiological procedures, such as mixing, homogenizing, serial dilution, and plate counting, may disrupt some of these clumps, while others may remain intact. A standard plate count procedure would therefore generate colonies that grow either from individual cells or from a clump of cells, hence the desirability of reporting such data in terms of colony forming units (cfu) rather than numbers of bacteria.

To obtain a representative sample from a bulk of liquid (eg, a milk churn, a tanker, a silo) would require significant effort to ensure that the liquid was intimately mixed before removal of a sample for analysis. As the volume of bulk liquid increases, the difficulty of ensuring effective mixing becomes ever more difficult. For a dry food material consisting of individual particles (eg, grain, nuts, flour), the difficulty of ensuring a truly representative sample is increased further, and when solid foods are examined (eg, meat, vegetables, compound food products), the impact of microbial distribution is likely to present yet more complexity. In such circumstances, and especially where growth of microorganisms may have occurred on or within the food, the importance of taking sufficiently large samples properly to assess the likely level of microbial contamination and/or the occurrence of specific microbes cannot be overstressed.[28,30] Similar situations pertain to surfaces, both of foods and process plant. Contamination of an unclean surface will result in opportunity for some microorganisms to grow if other environmental conditions are appropriate; at any specific temperature, some organisms may die, some will remain as single cells, while others will form microcolonies (Figure 60–1). If such a surface is subjected to ineffective cleaning and sanitation, some of those organisms will be killed, others will be sublethally damaged, and others (possibly within a micro-colony) may remain viable. Hence, the distribution of the organisms will change from random to a more complex distribution, indicating nonrandomness, such as the negative binomial distribution. Figure 60–2 illustrates the different distribution curves for Poisson,

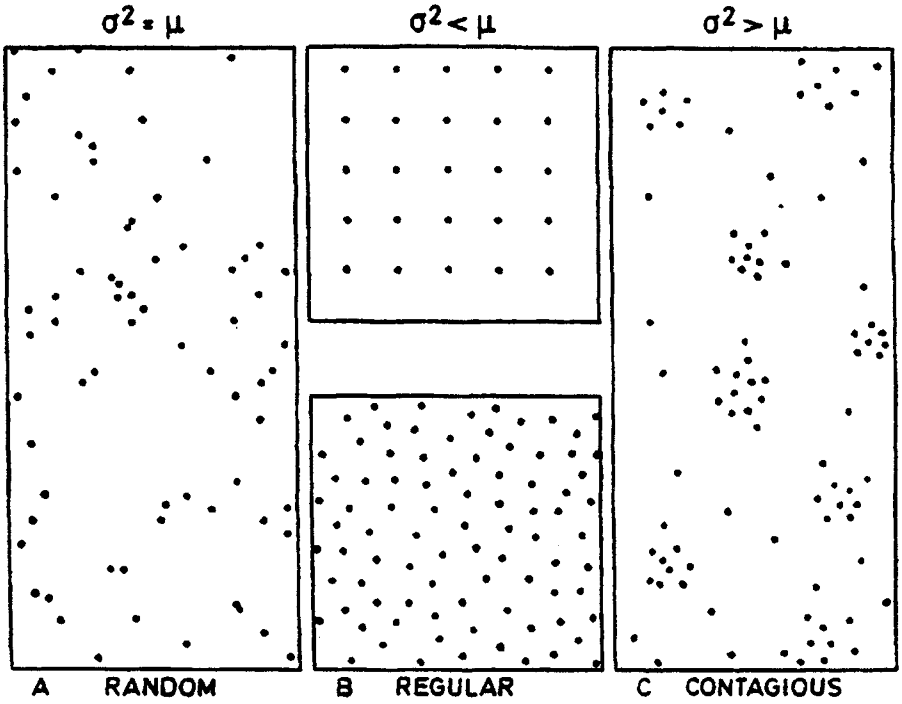
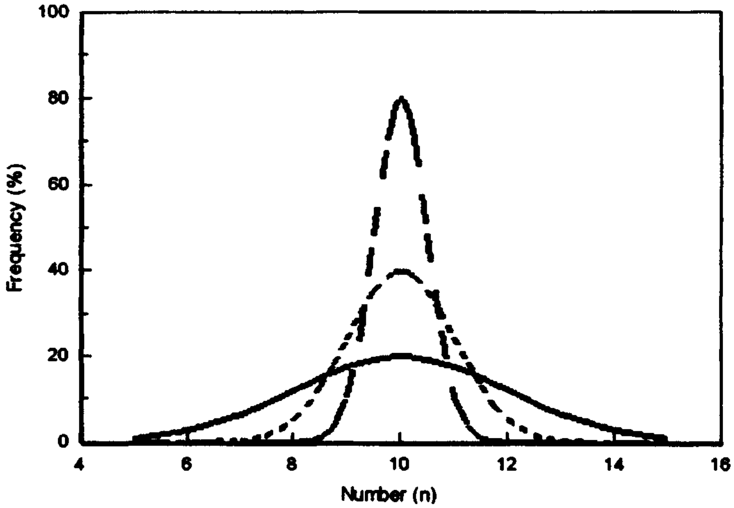
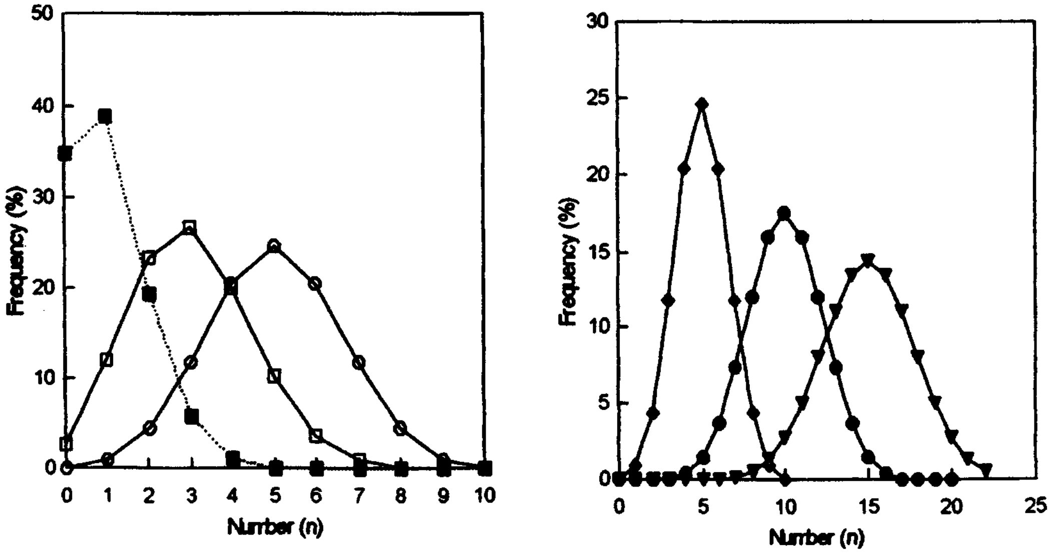


Figure 60–1 Illustration of Three Types of Spatial Distribution. (**A**) Random (ie, Poisson) distribution showing individual cells, (**B**) Regular (binomial) distribution (upper—ideal form, lower—nonideal form), (**C**) Contagious (eg, negative binomial) distribution showing presence of randomly distributed clumps and individual cells. *Source:* Adapted with permission from J.M. Elliott, *Some Methods for the Statistical Analysis of Samples of Benthic Invertebrates*, 2nd ed., Freshwater Biological Association Scientific Publication No. 25, 1977, Freshwater Biological Association.



A

Normal (Gaussian) distribution curves with a mean of 10 and standard deviations of ± 0.5 (- - -), ± 1.0 (.....) and ± 2.0 (——)



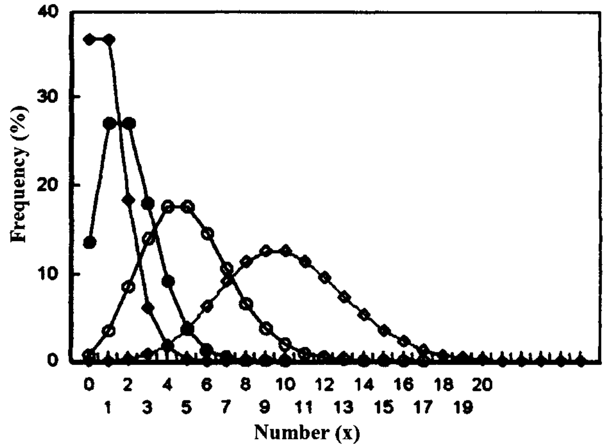
B

Binomial distribution curves based on the expansion of the term (p + q)ⁿ for various values of p and n, where p = probability that an event will occur, q = probability that an event will not occur = 1 - p, and n = number.

(i) $p = 0.1$, $n = 10$ (■), $p = 0.3$, $n = 10$ (□) and $p = 0.5$, $n = 10$ (○)
(ii) $p = 0.5$, $n = 10$ (◆), $p = 0.5$, $n = 20$ (●) and $p = 0.5$, $n = 30$ (▼)

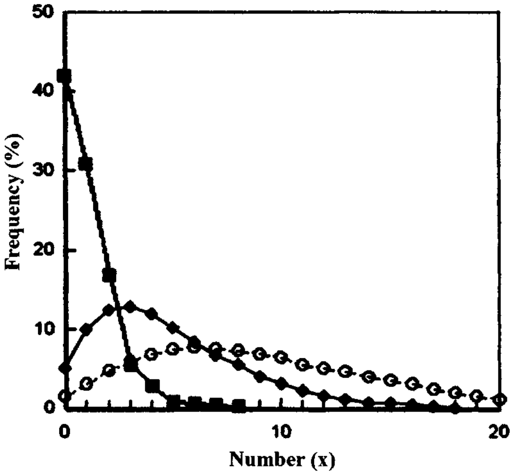
continues

Figure 60–2 Examples of Statistical Distribution Curves. (**A**) Normal (Gaussian), (**B**) Binomial.



C

Poisson distribution curves based on the expansion of $e^{-m}m^x/x!$, *where* e = *exponential,* m = *mean,* x = *number of random events and* $x!$ *means the factorial of* x, *for mean values* (m) *of* 1 (◆), 2 (●), 5 (○)and 10 (□)



D

Negative binomial curves derived from the expansion of $(q - p)^{-k}$ *for* $k = 3.0$ *and mean values* (μ) *of* 1(■), 5 (▲) and 10 (○)

Figure 60–2 continued (C) Poisson, (D) Negative Binomial

binomial, negative binomial, and "normal" distributions with low mean (average) values.

Why is it important to understand the nature of microbial distributions? First, if analytical data are to be compared, then it is essential that the individual sets of data come from populations having similar distribution characteristics. Second, most statistical tests of difference and significance are based on the concept of the "normal" or Gaussian distribution. Microbial and other data can be "normalized" by transformation such that the transformed data follow a distribution that approximates to "normal." Most colony counts yield data that, after transformation by the use of logarithms, conform approximately to "normal"—hence, the expression "a log normal distribution" (Figure 60–3). However, if a genuine random distribution occurs, the normalization of the data is best done by taking the square root of the original data value (ie, $\sqrt{x}$, where "x" is the value determined).[14]

The principal characteristics of some different statistical distributions are summarized in Table 60–1, and the interrelationship between the different distributions is illustrated schematically in Figure 60–4. The binomial distribution (which is important in defining sampling probabilities, qv) is characterized by having a population distribution variance smaller than the population mean value; by contrast, in the negative binomial distribution the variance is greater than the mean; and in the Poisson or random distribution, the mean and variance are equal. The Poisson distribution is a "limiting" form of the binomial distribution in which the probability (p) of an event occurring is very small (eg, the incidence of bacteria in a defined aliquot of liquid). The binomial and Poisson distributions are discrete or discontinuous distributions, whereas the normal is a continuous distribution. For further information on

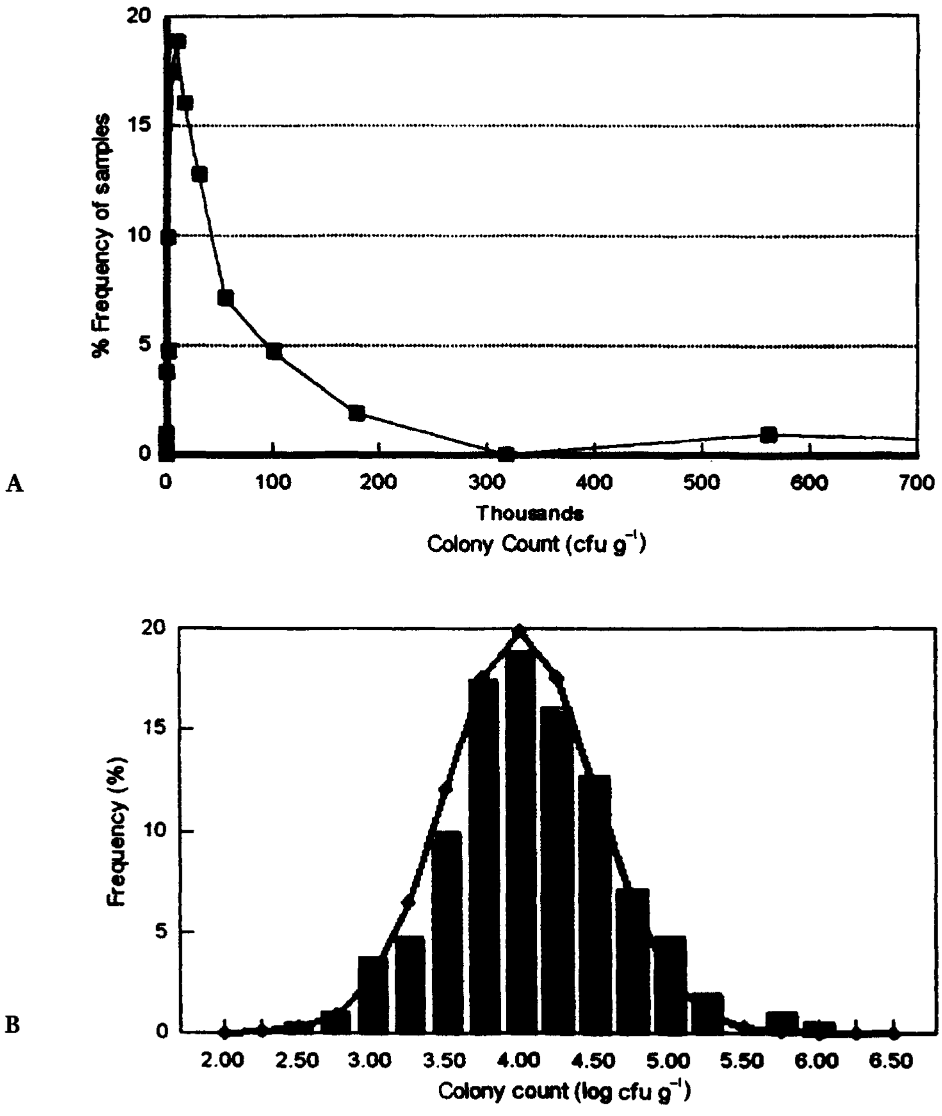


Figure 60–3 Distribution of Colony Counts from a Food Sample (A) before and (B) after logarithmic transformation. A shows the frequency distribution curve from replicate sample units plotted on a linear scale. Note the concentration of data points at the lower end of the ordinate. B shows the same data after logarithmic transformation plotted as a histogram and overlaid with a normal distribution curve.

Table 60–1 Some Characteristics of Continuous and Discrete Distribution Functions

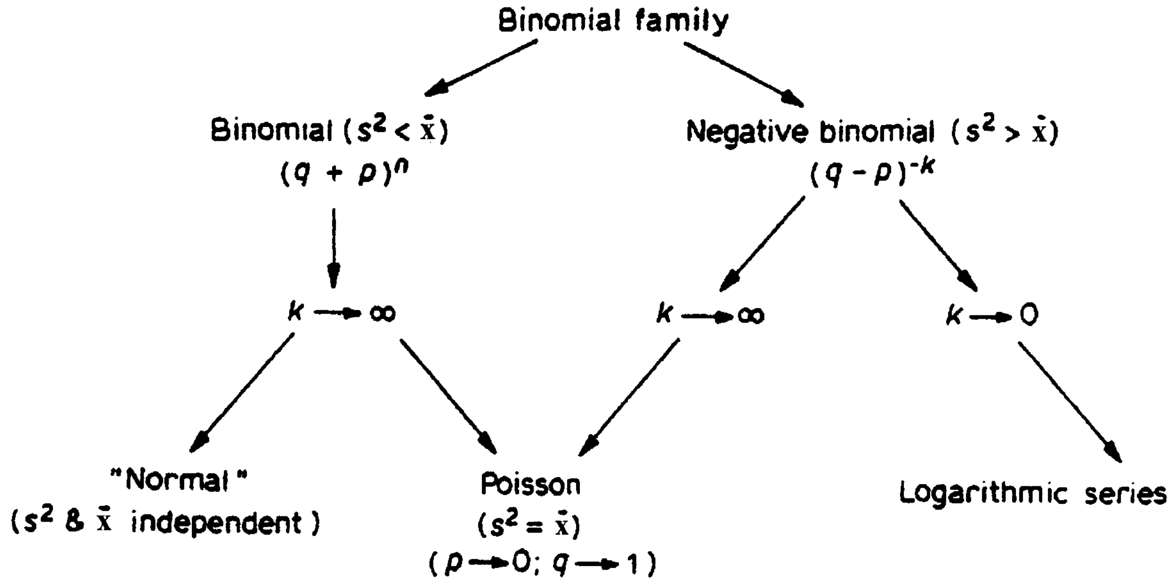
Name	*Domain*	*Probability Density Function f_x*	*Restriction on Parameters*	*Mean*	*Variance*
Normal (Gaussian)	$-\infty < x < \infty$	$(1/\sigma\sqrt{2\pi}) \exp [-\tfrac{1}{2} (x - m)/s^2]$	$-\infty < x < \infty$ $0 < \sigma < \infty$	μ	σ^2
Binomial	for $x = 0, 1, 2, \ldots, n$	$\binom{n}{x} p^x (1 - p)^{n-x}$	$0 < p < 1$ $q = 1 - p$	np	npq
Poisson*	for $x_n = 0, 1, 2, \ldots, \infty$	$e^{-m} . m^x / x!$	$0 < m < \infty$	$\lambda = m$	m
Negative binomial	$x_n = 0, 1, 2, \ldots, \infty$	$\binom{n + x - 1}{x} p^n (1 - p)^x$	$n \geq 0$ and $0 < p < 1$ ($p = 1 / Q$ and $1 - p = P/Q$)	nP	nPQ

*Limiting from a binomial as $p \rightarrow 0$ and $q \rightarrow \infty$

distributions, refer to standard statistical texts (eg, Bowker,[7] Rahman,[38] or Jarvis[28]).

To assess whether a set of microbial counts follows a particular distribution, it is necessary to test the hypothesis that the data conform to, for example, a Poisson distribution, by comparing the distribution frequency of the data with the expected distribution frequency based on the mean value and variance of the experimental data. The "goodness of fit" between the theoretical (expected) and actual data can be tested using a χ^2 test, where:

$$\chi^2 = \Sigma \frac{(O - E)^2}{E}$$



$\bar{x}$ = mean; s^2 = variance; p = probability of success at each trial; q = 1 - p; n = number of trials; k = distribution constant; → means tends to (e.g. p → 0 means as p tends to zero) and ∞ = infinity.

Figure 60–4 Inter-relationships between Frequency Distributions. *Source:* Reprinted with permission from B. Jarvis, Statistical Aspects of the Microbiological Analysis of Food, *Progress in Industrial Microbiology,* Vol. 21, p. 30, © 1989, Elsevier Applied Science.

and Σ means the sum of, E = the expected (theoretical) value, and O = observed value.

A simple method to assess conformity with a Poisson distribution, Fisher's Index of Dispersion test,[18] compares the actual and theoretical variances directly. An estimate of sample variance (s^2) is determined as the weighted mean of the sum of the squares of the difference ($x_n - \bar{x}$) of each individual result (x_n) from the mean result ($\bar{x}$); the "weighting" being introduced by dividing by the number of degrees of freedom (n–1) rather than the actual number of tests (n). The sample variance (s^2) is determined from the equation:

$$s^2 = \Sigma(x_n - \bar{x})^2 / (n-1)$$

The theoretical variance (σ^2) is given by the estimate of the mean $\bar{x}$ since, in a Poisson distribution, the population mean value (m) equals the population variance (σ^2). The Index of Dispersion (I)—the ratio of sample and theoretical variance—is determined from the equation:

$$I = \text{sample variance/theoretical variance}$$
$$= s^2 / \bar{x} = \Sigma(x_n - \bar{x})^2 / (n-1)\bar{x}$$

and is related to χ^2 by the expression: $\chi^2 = I\,(n-1)$

The significance of the extent to which I departs from unity can be determined from a table of χ^2 since $I\,(n-1)$ is approximated by χ^2 with ($n-1$) degrees of freedom, under the null hypothesis that the distribution does not diverge from Poisson. From tables of χ^2, or more approximately from Figure 60–5, agreement with a Poisson distribution is not disproved at the 95% probability level ($P > 0.05$) if the χ^2 value lies between the upper and lower significance levels for ($n-1$) degrees of freedom. If agreement is perfect, I = 1 and $\chi^2 = (n-1)$. More details of this and other tests for goodness of fit are given by Jarvis.[28]

60.1.2 Reproducibility and Precision of Microbiological Test Methods

The quality of data produced by any type of analysis is effectively measured in terms of the accuracy, repeatability, and reproducibility of the method. *Accuracy* is a measure of the quality of the test method (ie, of its ability to provide results equal to the true value). Deviations from the true value are referred to as method bias. *Repeatability* is the same as *random error* and is a measure of the difference between two single results found on identical material by one analyst using identical test conditions. The *repeatability value* (r_{95}) is the absolute value of the difference between two single results determined as above that will be found in 95% of analyses. *Reproducibility* is a measure of the difference between two single results obtained on identical material reported by two laboratories using identical test methods. The *reproducibility value* (R_{95}) is the absolute value of the difference in such interlaboratory results that will be found in 95% of tests. While it is quite normal for chemical and physical methods to cite repeatability and reproducibility parameters, they are rarely found in microbiological methods. However, interlaboratory trials of methodology such as those undertaken by the International Dairy Federation[37] are seeking to calculate both the interlaboratory and the intralaboratory errors and hence both the reproducibility and repeatability of the methods.

Intrinsic and extrinsic errors associated with serial dilution and colony count procedures include the accuracy of measuring the quantity of analytical sample, the accuracy of pipettes and pipetting, the accuracy of the diluent volumes dispensed, plating and incubation effects, colony counting and mathematical errors, and microbial distribution effects.[28] Assuming a random distribution of colonies in an agar plate, then the limiting precision (*Confidence Interval [CI]*) of the colony numbers can be derived from the actual count. As shown above, for data conforming to a Poisson distribution, the variance (s^2) is equal to the mean value ($\bar{x}$); hence:

$$s = \sqrt{\bar{x}}$$

By definition, approximately 95% of all replicate results will be expected to lie within the range $\bar{x} \pm 2s$. If the number of colonies counted totals 400, then the 95% precision of the colony count will range from $400 \pm 2 \times 20$ (ie, from 360 to 440). The precision of the count as a percent of the mean will be $\pm (40 \times 100)/400$ (ie, ± 10%) (Table 60–2). If the total number of colonies counted is 400, the limiting precision, at 95% probability, is approximately ± 10%, but if only 40 colonies are counted, the precision of the count reduces to ± 37% and at 10 colonies the precision is only ± 60%. However, if the colony distribution is not random, for instance if it shows evidence of contagion (*qv*), the 95% limiting precision (ie, the 95% confidence limits) will be much larger and significantly asymmetric.

The overall compound standard error of the mean count can be derived using the equation:

$$\textbf{Total SE}_{\text{mean}} = \pm\sqrt{[(A)^2 + (B)^2 + (C)^2 + \ldots (X)^2 + (Y)^2 + (Z)^2.]}$$

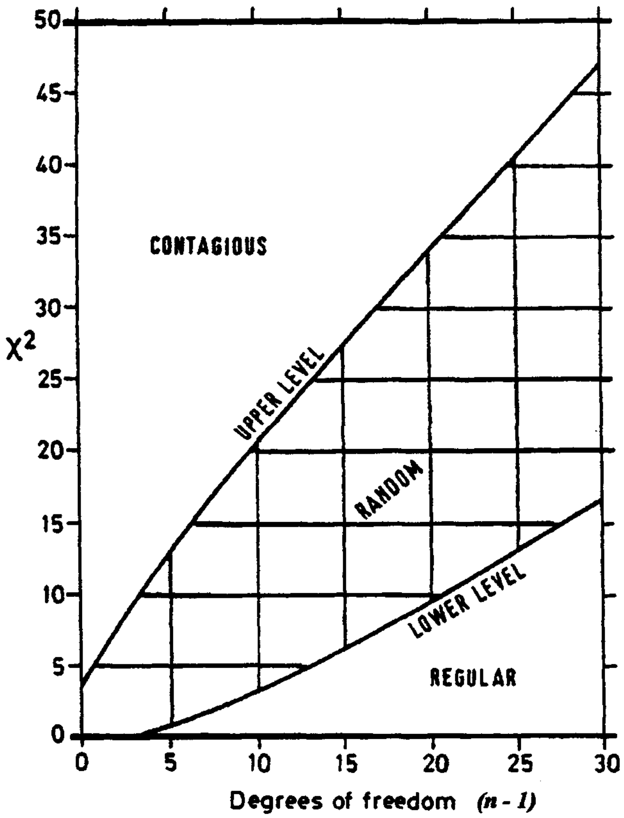


Figure 60–5 Goodness of Fit Test for Conformity with a Poisson Distribution. The χ^2 value is determined as described in the text for n -1 degrees of freedom, where n = the number of replicate counts made. The upper and lower limits for conformance with a Poisson distribution are set at p = 0.025 and p = 0.975, respectively. *Source:* Adapted with permission from J.M. Elliott, *Some Methods for the Statistical Analysis of Samples of Benthic Invertebrates*, 2nd ed., Freshwater Biological Association Scientific Publication No. 25, © 1977, Freshwater Biological Association.

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where A = SE$_{\text{mean}}$ due to bacterial distribution, B = SE$_{\text{mean}}$ due to sampling errors, C = SE$_{\text{mean}}$ due to dilution errors, and.... X, Y, and Z refer to the errors associated with other aspects of the analysis (eg, counting).

Hence if the 95% limiting precision of sampling is ± 10%, the error associated with colony distribution is ± 30% and the error associated with dilution ± 10%, the overall precision of the count would be ± $\sqrt{[30^2 + 10^2 + 10^2]}$, ie, $\sqrt{[1100]}$ = ± 33.2%. Hence in 19 of 20 tests, a count of 45 colonies on the 10^{-6} dilution would indicate a microbial level of (45 ± 14) × 10^6 cfu ml^{-1} (ie, the count of 45 × 10^6 cfu would indicate a probable level of contamination within the range 30 × 10^6 to 60 × 10^6 cfu ml^{-1}; however, on average 1 in every 20 tests might be expected to give a true colony count outside that range. If the counts are transformed into their logarithms, the log cfu of 7.65 has a 95% CI ranging from 7.49 to 7.77. It should be noted that this range is now nonlinear as a result of the transformation. It is important to note, in the example above, that the potential error due to bacterial distribution (± 30%) is responsible for most of the combined error (± 33.2%), assuming reasonably good laboratory technique.[28]

Tests on replicate samples will each have their own limits of reproducibility based on the distribution of the mean counts of each replicate analysis. Experience suggests that on average, 95% of the mean log cfu counts will lie within ± 0.5 log units for a plate count on unstressed organisms, such as those from a broth culture. For plate counts on food materials, it can be expected that the range of counts lying within ± 2 standard deviations of the mean will often be much wider; for instance, replicate plate counts on bowl-chopped beef (Table 60–19) showed a mean log count of 6.48 with a variance of 0.075. This gives a standard deviation of the count of ± 0.274 log units such that approximately 95% of the counts would lie within ± 2 × 0.274 = ± 0.55 log units.

Provided that the individual log colony counts are distributed normally and that the means of replicate series of counts are themselves normally distributed, then on 95% of occasions the mean count indicates a true mean count lying within the range ± t × SE$_{\text{mean}}$, where t is a value, derived from tables of Student's t distribution, which is dependent on the required probability and the number of replicate tests (n). The SE$_{\text{mean}}$ is derived from the standard deviation (s) using the equation: SE$_{\text{mean}}$ = $s / \sqrt{n}$. In the example quoted above, for bowl-chopped thawed beef, the SE$_{\text{mean}}$ would be ± $0.274/\sqrt{20}$, ie, ± 0.06; and at 95% probability the value of t = 2.060, for n -1 (= 19) degrees of freedom. Hence, the 95% CI of the estimate of the population log mean count is given by 6.48 ± 0.06 × 2.06 (ie, 6.36 to 6.60). However, thawed beef (Table 60–19) had a mean log count of 5.93 with a variance of 0.334, such that 95% of log counts would be expected to lie within the range 5.93 ± 1.16 (ie, 4.77 to 7.09). The 95% CI around the mean is 5.93 ± 0.27, or a range from 5.67 to 6.20 log units. The literature contains examples[31] of much larger variances in log colony counts on foods with smaller numbers of replicate tests such that 95% CI

Table 60–2 Approximate 95% Confidence Limits for Numbers of Colonies Counted, Assuming Agreement with a Poisson Series

Mean Number of Colonies Counted	*Standard Deviation (s)*	*Approximate 95% limit (± 2 s)*	*95% Confidence Limits*	*95% Confidence Limits As % of Mean (to nearest %)*
500	± 22.4	± 45	455–545	± 9
400	± 20.0	± 40	360–440	± 10
320	± 17.9	± 36	284–356	± 11
200	± 14.1	± 28	172–228	± 14
100	± 10.0	± 20	80–120	± 20
80	± 8.9	± 18	62–98	± 22
50	± 7.1	± 14	36–64	± 28
40	± 6.3	± 13	27–53	± 32
30	± 5.5	± 11	19–41	± 37
20	± 4.5	± 9	11–29	± 45
16	± 4.0	± 8	8–24	± 50
10	± 3.2	± 6	4–16	± 63
6	± 2.4	± 5	1–11	± 82

values, for mean log colony counts, in excess of ± 0.5 log units are commonplace. Any circumstances leading to a high variance (eg, nonrandom microbial distribution, sublethal damage, use of differential culture media) is likely to result in large CIs (ie, the mean values will have lower precision than the examples cited above). Such counts often will be used to make a decision as to the acceptability of a batch of product!

The CIs of most probable number (MPN) tests generally are wider than those obtained for colony count data; however, the extent of the CI will be dependent on a number of factors, including the dilution ratio and the number of replicate tests undertaken. For instance, from Table 60–3, a series of tests using 5 replicates at 10-fold serial dilutions of 1g, 0.1g, and 0.01g samples giving results of 3+, 2+, and 1+ would indicate an MPN of 1.7 organisms g^{-1} with a 95% CI of 0.7 to 3.9 organisms g^{-1}. It should be noted that this CI is asymmetrical about the MPN value determined. MPN counts can be made more accurate by using more replicates or by choosing a combination of replicates that will provide an estimate complying with a particular critical count level.[5] CIs for MPN data can be obtained from tables[15] or can be calculated from published equations.[12]

60.1.3 Acceptance Sampling by Assessment of Attributes or Variables

Whenever a decision is to be made about the acceptability of a product, it is necessary to first define the criteria against which that decision will be made. In the case of microbial contamination, the criteria may relate to numbers and/or types of organism that may pose an unacceptable risk to the well-being of the consumer or the quality of the product. For instance, the acceptability criteria for a product may include a standard plate count of aerobic mesophilic organisms, the absence of indicator organisms, and the absence of specific pathogens. Examples of such criteria are illustrated in Table 60–4 taken from The International Commission on Microbiological Specifications for Foods (ICMSF);[24] many other examples of microbiological criteria have been published,[2,3,4,13] although not all define proper sampling plans;[2,3,4] see also Chapter 65.

As an example of Attributes sampling, the ICMSF[24] plan for dried milk powder (Table 60–4) has limits for coliforms of $m = 10$ and $M = 100$. This is a three-class Attributes sampling plan (qv) where counts $\leq m$ are deemed to be acceptable, counts $> M$ are unacceptable, and counts $> m$ but $\leq M$ are deemed to be marginally acceptable. The defined limits m and M are therefore criteria against which decisions can be taken. The sampling plan also defines the number of sample units (n) to be tested (in this case $n = 5$) and provides a parameter (c) that defines the number of marginally acceptable sample results that can be tolerated ($c = 1$ in this case). The stringency of the sampling plan is defined principally by the values of n and c and is discussed in more detail below.

Other criteria for the dried milk powder include two-class plans for *Salmonella* spp. for various situa-

Table 60–3 Examples of 95% and 99% Confidence Limits for Category 1, Most Probable Number Values Based on Testing 5 Replicates at Each of Three 10-fold Dilution Levels

Number of Positive Results with Inocula of				*Confidence Limits at P = 0.95*		*Confidence Limits at P = 0.99*	
1g	0.1g	0.01g	*MPN g*$^{-1}$	Lower	Upper	Lower	Upper
0	0	1	0.18	0.00	0.65	0.00	0.93
1	0	1	0.40	0.07	1.00	0.02	1.40
1	2	0	0.61	0.18	1.40	0.09	2.10
2	0	0	0.45	0.08	1.40	0.04	2.10
2	1	0	0.68	0.19	1.70	0.10	2.30
3	0	0	0.78	0.21	2.20	0.12	2.80
3	0	1	1.1	0.4	2.2	0.2	2.9
3	2	1	1.7	0.7	3.9	0.5	5.1
3	3	0	1.7	0.7	3.9	0.5	5.2
4	0	0	1.3	0.4	3.4	0.3	4.4
4	2	1	2.6	1.0	6.6	0.7	9.4
4	3	1	3.3	1.0	6.6	0.7	9.4
4	4	0	3.4	1.3	10.0	0.9	14.7
5	0	0	2.3	0.7	6.6	0.5	9.4
5	0	1	3.1	1.0	6.6	0.7	9.4
5	2	0	4.9	1.5	14.9	0.9	20.0
5	3	1	11	3	24	2	32
5	3	2	14	5	35	3	45
5	4	0	13	3	35	3	45
5	4	1	17	6	39	4	51
5	4	3	28	10	70	6	92
5	5	0	24	7	70	4	92
5	5	3	92	23	253	15	338

Source: Data from J.C. de Man, MPN Tables Corrected, *European Journal of Applied Microbiology*, Vol. 17, pp. 311–305, © 1983.

tions. In the case of product for high-risk situations, where the risk is likely to be increased following reconstitution of the dried milk, the plan requires $n = 60$, $c = 0$, and $m = 0$ with an analytical unit of 25g: in this instance, the acceptability criterion is that salmonellae should not be detected in 60 × 25g samples using a defined analytical method. The choice of analytical method is determined according to the purpose of the test and may include resuscitation stages. In these examples of microbiological Attributes sampling, decisions are based on the defined acceptability criteria and the standard statistical considerations applicable to any sampling protocol.

However, the concept of *Variables sampling* provides an alternative approach that uses more fully the data available from the analysis of replicate samples.[28,32] The data (eg, as cfu or log cfu g^{-1}) can be analyzed statistically to generate a value for the mean colony count (contamination level) together with a value for statistical variance around that mean value. Such data can then be compared with criteria defined in a different manner from those used in assessment of Attributes and use all the available data rather than making individual judgments on each test result. Variables sampling procedures more normally are used in chemical and physical analyses but have a role in modern food microbiology. A fuller exposition on Attributes and Variables sampling is given below (see Sections 60.2.2 and 60.2.3).

60.1.4 HACCP and Statistical Process Control

If the Hazard Analysis Critical Control Point System (HACCP), which is discussed elsewhere in this

Table 60–4 Sampling Plans and Recommended Microbiological Limits for Dried Milk

	Case			*Number of Sample Units*	*Number of Results Allowed To Exceed m*	*Limit g^{-1}*	
*Test**	*Basis for Test*	*Potential Change in Hazard†*	*Plan*	(*n*)	(c)	*m*	*M*
Aerobic plate count	Utility	No change	3-class	5	2	3×10^4	3×10^5
Coliforms	Indicator of low potential health hazard	No change	3-class	5	1	10	100
Salmonella (normal, routine)	Moderate hazard, direct, potentially extensive spread	Reduced hazard	2-class	5	0	0‡	
		No change	2-class	10	0	0	
		Increased hazard	2-class	20	0	0	
Salmonella for high-risk population	Severe direct health hazard	Reduced hazard	2-class	15	0	0	
		No change	2-class	30	0	0	
		Increased hazard	2-class	60	0	0	

* Based on specified microbiological methods

† Dependent on the conditions in which the food is expected to be handled and consumed after sampling

‡ None detected in each sample unit of 25g

Source: Adapted with permission from ICMSF, Micro-organisms in Foods. Vol. 2. *Sampling for Microbiological Analysis: Principles and Specific Applications*, 2nd ed., © 1986, University of Toronto Press.

book (Chapter 58), is to be effective, it is necessary to use appropriate procedures both to monitor and then to verify that a process is in control. The microbiological considerations will be explored in conjunction with the use of Statistical Process Control (SPC), a concept used to monitor production processes for key product parameters, which can also be applied to microbiological testing. Many data are never used effectively because once they have been filed, they are rarely seen again. In SPC, data are used on a continuous basis to assess whether unacceptable trends are developing over a period of time. The concept of SPC can be applied also to check that laboratory analyses are themselves "in control;" both aspects will be discussed in more detail later in this chapter in Section 60.4.

60.2 STATISTICAL BASIS OF SAMPLING PLANS

60.2.1 Characteristics of Statistical Distributions

Reference has been made previously (Section 60.1.1) to some of the different types of statistical frequency distributions, especially Poisson, binomial, negative binomial, and normal distributions and the relationships between them (Table 60–1, Figure 60–4). A frequency curve describes the probability that a particular event will occur in a given set of circumstances. For instance, if we flip a number of coins, we can predict the probability of getting a particular combination of heads and tails. Since the 2 sides of a coin are different, there is a 50% chance that when a single coin is spun, we will get either a head or a tail. If 2 coins are spun, the probabilities of getting 0, 1, or two heads are 25%, 50%, and 25%, respectively. Similarly, if we have 4 coins, the probabilities for obtaining 0, 1, 2, 3, or 4 heads are 6.75%, 25%, 37.5%, 25%, and 6.75% respectively; and so on for larger numbers of coins. The sum of all probabilities *must* equal 100% (ie, $P = 1$).

This is analogous to estimating the probability that a randomly drawn sample of n units will contain 1, 2, 3, ... n defective units at any given incidence of actual defective units in the population from which the samples were drawn. Mathematically, we can derive the probability (P_x) of obtaining any given number (x) of defective units out of a sample of n units, from the general equation for the binomial distribution:

$$P_{(x)} = \frac{n!\,p^x q^{(n-x)}}{x!(n-x)!}$$

where n = the number of sample units, x is the number of defective units, p is the true probability of occurrence of defective units in the lot, q is the true probability of occurrence of acceptable units in the lot, and ! means the "factorial value." That is, $n!$ means the multiple of: $\boldsymbol{n}\,(\boldsymbol{n}-1)\,(\boldsymbol{n}-2)\,(\boldsymbol{n}-3)\ldots 1$. By definition, $\boldsymbol{q} = (1-\boldsymbol{p})$.

Hence, for a lot containing 20% defective units ($p = 0.2$ and $q = 0.8$), the probability of detection of 1, 2, 3, or more defectives in a sample of size n can be calculated. The probability of occurrence of no defectives in a sample of 10 units, assuming always that the samples are fully representative of the lot and that the methodology is "perfect," is given by:

$$\boldsymbol{P}_{(x=0)} = 10!\,(0.2)^0(0.8)^{10} / 0!\,(10-0)! = 10!\,(0.8)^{10} / 10! = 0.8^{10} = 0.107$$

Similarly, the probability of occurrence of 1 defective in 10 sample units is given by :

$$\boldsymbol{P}_{(x=1)} = 10!\; 0.2^1\; 0.8^{(10-1)} / 1!\,(10-1)! = 0.268$$

and the probability of occurrence of 2 defectives in 10 sample units is given by:

$$\boldsymbol{P}_{(x=2)} = 10!\; 0.2^2\; 0.8^{(10-2)} / 2!\,(10-2)! = 0.3024$$

Since the proportion of acceptable samples (q) is given by ($1-p$), where p is the proportion of defective units, then the probability of occurrence ($P_{(x>0)}$) of 1 or more defective units in a sample can be derived from:

$$\boldsymbol{P}_{(x>0)} = \{\, 1-\boldsymbol{P}_{(x=0)} \}$$

Examples of the probability of occurrence of various levels of defective units in a sample size of 10 units for lots having different levels of incidence of actual defective units from 0.1% to 50% are shown in Table 60–5. Similarly, Table 60–6 illustrates the probability of occurrence of defective units according to sample size in lots having a 10% incidence of actual defective units.

From Table 60–5, it can be seen that the probability of occurrence (P_x) of 0 defectives in a sample of 10 units decreases from 0.99 when the true incidence of defectives in a lot is 0.1%, to 0.35 with 10% true defectives and 0.11 with 20% defectives. Conversely, the probability of occurrence of one or more defectives is 0.01, 0.09, 0.65, and 0.89 for lots having 0.1%, 1.0%, 10%, and 20%, respectively, if the sample consists of 10 units. Similarly, from Table 60–6, the probability of occurrence of one or more defective units from a lot having 10% true defectives increases with increasing sample size from 0.41 for 5 samples, to 0.65 for 10 samples, to 0.88 for 20 samples, and 1.00 for 100 samples. This means that unless the incidence of defectives is high, the likelihood of detecting defective materials in a lot is very low, unless large numbers of sample units are examined.

60.2.2 Statistical Aspects of Two-Class Attributes Sampling Plans

A two-class sampling plan defines the criteria for acceptance of a sample unit (eg, salmonellae not detected in 25g of product or standard plate count not greater than 10^6 cfu g^{-1}), the number of samples to be tested (*n*), and whether any defective (ie, nonacceptable) samples can be tolerated (designated *c*). In determining the sampling plan, due consideration must be given to the concepts of producer's risk and consumer's risk (see Appendix 60–A for definitions).

The data on the probability of acceptance of a lot containing defective units (Tables 60–5 and 60–6) can be presented as a series of cumulative probabilities in Operating Characteristics (OC) Curves (Figures 60–6 and 60–7). These illustrate the probabilities of acceptance, or rejection, of samples with particular levels of occurrence of defective sample units according to both the true incidence of defectives and the numbers of sample units tested. In Figure 60–6, two specific points are identified as the acceptance quality level (AQL) and the rejectable quality level (RQL) (also known as lot tolerance percent defectives; LTPD). The AQL indicates the probability of rejection of good lots. An acceptance parameter α (which should be close to zero: in the figure the value used was 0.05) is defined giving an AQL that is numerically equivalent to $1-\alpha$ (eg, 0.95 in Figure 60–6). For the illustrated data, the producer's risk would have a 95% chance of accepting lots with up to 9% defective units and a 5% chance of rejecting lots with < 9% defectives (ie, good product). At the other extreme, the parameter RQL defines consumer's risk, which is determined through a parameter β. In the example in Figure 60–6, β is set at 0.10, so that there would be a 10% chance that the accepted lots would contain 45% defective units and a 90% chance of rejecting lots with > 45% defective units. In setting a sampling plan, it is necessary to decide on the relative risks that should be applied to producer's and consumer's risk to establish values for α and β. Table 60–7 shows the proportion of defective units likely to be accepted or rejected at $P_a = 0.95$ and $P_r = 0.90$, respectively, for various values of *n* and *c*.

Figure 60–7 shows OC curves for various two-class Attributes plans with sample sizes of 5, 10, and 20 units. With a plan that allows no defective units (ie, $c = 0$) and examines 20 sample units (ie, $n = 20$),

Table 60–5 Probability of Occurrence of 0, 1, 2, ... 10 Defectives in 10 Sample Units from Lots with Different True Incidences of Defective Units (Based on Binomial Distribution)

True Incidence (p) of Defective Units in the Lot	*Probability (P_x) of Detecting the Following Number (x) of Defectives in 10 Sample Units from a Lot*										
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0.1%	0.99	0.01	<	<							
1%	0.90	0.09	<0.01	<	<						
5%	0.60	0.32	0.08	0.01	<0.01	<	<				
10%	0.35	0.39	0.19	0.06	0.01	<0.01	<	<			
15%	0.20	0.35	0.28	0.13	0.04	0.01	<0.01	<	<		
20%	0.11	0.27	0.30	0.20	0.09	0.03	<0.01	<	<		
25%	0.06	0.19	0.28	0.25	0.15	0.06	0.02	<0.01	<	<	
30%	0.03	0.12	0.23	0.27	0.20	0.10	0.04	0.01	<0.01	<	<
40%	<	0.04	0.12	0.22	0.25	0.20	0.11	0.04	0.01	<0.01	<
50%	<	0.01	0.04	0.12	0.21	0.25	0.20	0.12	0.04	0.01	<

< = less than 0.001

Table 60–6 Binomial Probability of Detecting 0, 1, 2, ... 10 Defective Units)(with Increasing Number of Sample Units from a Lot Having a True Incidence of 10% Defective Units

Number of Sample Units (n) Tested	*Probability of Detecting the Following Number of Defective Units (x) from a Lot with a True Incidence of 10% Defective Units*										
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
5	0.59	0.33	0.07	<0.01	<	<					
10	0.35	0.39	0.19	0.06	0.01	<0.01	<	<			
15	0.21	0.34	0.27	0.13	0.04	0.01	<0.01	<	<		
20	0.12	0.27	0.29	0.19	0.09	0.03	0.01	<0.01	<	<	
25	0.07	0.20	0.27	0.23	0.14	0.07	0.02	<0.01	<0.01	<	<
50	<0.01	0.03	0.08	0.14	0.18	0.19	0.15	0.11	0.06	0.03	0.02
100	<	<	<0.01	<0.01	0.02	0.03	0.06	0.09	0.12	0.13	0.13

< = less than 0.001

the probability of accepting a lot containing 10% defectives is about 0.12, whereas for a lot containing 1% defectives, the probability of acceptance is about 0.95. If the sample size is reduced to 10 units, the probability of accepting a lot with 10% defectives increases to 0.35. However, if the sampling plan permits the acceptance of defective units, then for a sample plan of $n = 10$, $c = 2$, the probability of accepting a lot with 10% defectives increases to 0.93; whereas a plan of $n = 5$, $c = 2$ would accept 10% defectives with a probability of 0.98. The discriminatory power of a sampling plan is therefore dependent on both the number of sample units tested, and whether it is permissible to accept one or more defective units in the sample units tested. The greater the number of sample units tested, the higher the stringency of the test; the lower the numbers tested and the acceptance of defective units reduces significantly the stringency of the plan. Table 60–8 compares the OC characteristics of several ICMSF two-class sampling plans.

It is reasonable to use a sampling plan based on the *binomial distribution* for relatively small numbers of samples provided that the lot size is large compared with the number of sample units taken for testing. However, when the number of sample units is large relative to the number of defectives (which is itself large relative to zero), it may be more useful to apply the normal distribution. If the incidence of sample defectives is very low, it may be more appropriate to derive the sampling statistics using the *Poisson distribution*. Details of the use of these distributions can be obtained from standard statistical texts;[8,35] recommended sampling plans have been published for single-, double-, and multiple-sampling schemes for various lot sizes up to and above 500,000 units.[9]

It should be recognized that OC curves also can be used to answer questions such as, "What is the probability of detecting randomly distributed salmonellae if *n* sample units are tested and the lot is likely to be contaminated at a maximum given level of salmonellae per kg?" *Assume that salmonellae are randomly distributed in a product (unlikely in practice), that 20 sample units are tested and that no salmonellae are detected in the tests.* Then, even though no salmonellae are found, Figure 60–7 shows that there is still a 95% chance that the product lot would be accepted with a true incidence of defective sample units (i.e., those containing salmonellae) of 0.26% (*ca* 1 in 400); similarly, there is a 50% chance that the lot would be accepted if the true incidence of defective sample units is 3.4% and a 10% chance that the lot would be accepted if the true incidence of defective sample units is 10.9%. A mathematical equation to derive maximum contamination levels from replicate tests is given later (see Section 60.5.2).

60.2.3 Double Sampling Plans

The stringency of a sampling plan can be increased by increasing the number of sample units (*n*) to be tested (eg, from 10 to 30 or more) and by decreasing the number of sample units of unacceptable quality (*c*), which it is possible to accept for the purpose of the plan. Rather than increasing the number of

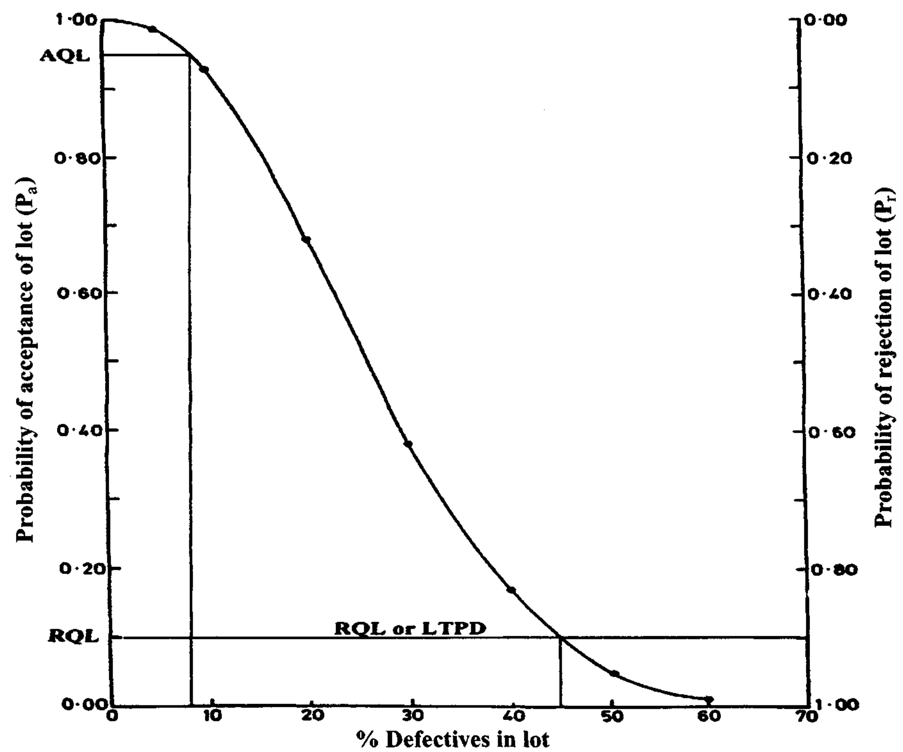

Figure 60–6 Operating Characteristics Curve Showing an Acceptable Quality Limit (AQL) of 9% Defective Units at P_a = 0.95 and a Rejectable Quality Limit (RQL, or LTPD) of 45% Defective Units at P_r = 0.90

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sample units in totality, it is sometimes advocated that sample units should be drawn in two stages, such that there are two interlinked sampling plans, as illustrated below with reference to a lot having 5% defectives:

Stage 1 $n_1 = 20$, $c_{1a} = 0$, $c_{1b} = 1$	***Accept*** with 0 defective sample units ***Repeat*** analysis, on second set of sample units, if 1 defective sample unit ***Reject*** if > 1 defective sample unit
Stage 2 $n_2 = 20$, $c_2 = 0$	***Accept*** with 0 defective sample units only
Overall plan: $n = 40$, $c = 1$	***Accept*** if $\not>$ 1 defective sample unit is found in the first sample set and none in the second set.

In the first stage, if no defective is found in 20 samples from a lot having 5% actual defectives, the probability of accepting the lot ($P_{x<1}$) would be 0.36. However, if the lot was considered to be acceptable with 1 positive from the 20 samples tested in the first analysis, the probability of accepting the lot with 5% defects would rise to 0.74. In double sampling, the finding of a single defective in the first series would require a second set of 20 samples to be tested. Assuming that no positive was detected in this second series, then the lot would be accepted with P_a = 0.36 for the second series of 20 samples. Overall, it might be considered that the P_a for the lot could be determined from the quotient of the probabilities for accepting the two sets each of 20 samples, for example,

$$P_{x\le1} = P^{1}_{x\le1} \times P^{2}_{x\le0} = 0.74 \times 0.36 = 0.27.$$

This is not correct.

If the second set of samples is drawn, the sample plan must then be considered to be $n = 40$, $c = 1$ which, for a true incidence of 5% defective units, would accept the lot with a P_a = 0.40. Table 60–9 illustrates the difference in P_a values for some single- and double-sampling plans with AQLs ranging from 1.0 to 10.0.

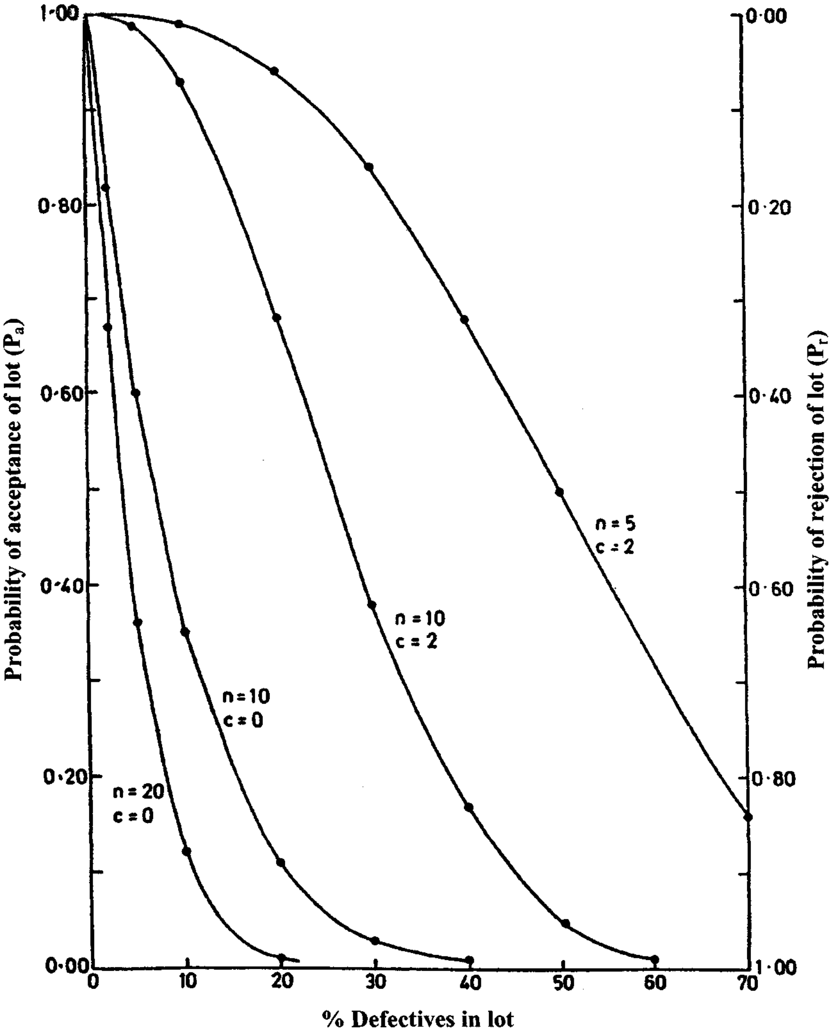

Figure 60–7 Operating Characteristics (OC) Curves for Two-Class Sampling Plans with $n = 5$, 10, or 20 and $c = 0$ or 2. *Source:* Reprinted with permission from B. Jarvis, Statistical Aspects of the Microbiological Analysis of Food, *Progress in Industrial Microbiology,* Vol. 21, © 1989, Elsevier Applied Science.

60.2.4 Statistical Aspects of Three-Class Attributes Sampling Plans

The introduction of three-class sampling plans was predicated on the recognition that quantitative estimation of microbial numbers by plate count and similar procedures has a relatively low accuracy and reproducibility. Factors affecting such data include not only technical inaccuracies involved in laboratory analysis but also the distribution of microorganisms in the original food sample.[28]

The three-class plan identifies the number of sample units (n) to be tested; a value (m) for acceptable colony counts, a value (M) for the upper limit of acceptability of counts, and a value (c) for the number of counts allowed to fall between m and M. Hence, a three-class plan might be:

$$n = 5 \quad c = 2 \quad m = 10^4\,g^{-1} \quad \text{and} \quad M = 10^5\,g^{-1}$$

This plan states that of five sample units tested, ideally all should give counts equal to or less than 10^4 cfu g^{-1} but not more than two sample units could have counts > 10^4 but ≤ 10^5 cfu g^{-1}; none must have counts > 10^5 cfu g^{-1}. However, whereas the two-class plan is normally based on the binomial distribution, the three-class plan is based on the trinomial distri-

Table 60–7 Incidence of Defective Units in a Lot That Would Have a 95% Chance of Acceptance (P_a) and a 90% Chance of Rejection (P_r) by Different Two-Class Sampling Plans Assuming All Replicate Tests Are Negative

	% Defectives in a Lot When			
	c = 0		*c = 1*	
Number of Sample Units (n)	*P_a = 0.95*	*P_r = 0.90*	*P_a = 0.95*	*P_r = 0.90*
5	1.0	37	7.6	58
10	0.5	25	3.5	39
20	0.3	12	1.8	20
30	0.2	8	1.2	13
50	0.1	5	0.7	8
100	0.05	2.5	0.35	4
150	0.04	1.7	0.23	2.6
200	0.03	1.2	0.18	2.0
500	0.01	0.5	0.07	0.8

Source: Adapted with permission from B. Jarvis, Statistical Aspects of the Microbiological Analysis of Food, *Progress in Industrial Microbiology*, Vol. 21, p. 78 © 1989, Elsevier Applied Science.

bution.[10] No colony count should exceed the upper level (M) so that the maximum acceptable number of defectives (c_M) is zero; but the sample plan will accept some marginal defectives (c_m) in the total number of samples tested (n). Hence the acceptable proportion of defective units is given by $p_M = 0$ and the acceptable proportion of marginal defective units is given by p_m. The probability for acceptance (P_a) of a lot of product can be derived from a simplification of the trinomial expansion, for values of i from 0 to c_m:

$$P_a = \sum_{i=0}^{c_m} \binom{n}{i} p_o^{n-i} p_m^i$$

where P_0 = proportion of good units, p_m = proportion of marginally defective units, n = number of sample units tested, and c_m = number of marginally defective units acceptable under the plan. This has the same form as that of a binomial expansion but it is not a general binomial since $p_0 + p_m \neq 1$. Values of P_a can be calculated from the equation:

$$P_a = \left\{ \sum_{i=0}^{c_m} \binom{n}{i} \left(\frac{p_0}{p_0+p_m}\right)^{n-i} \left(\frac{p_m}{p_0+p_m}\right)^{i} \right\} (p_0+p_m)^n$$

The expression inside the braces {} is a cumulative binomial term that can be read from standard tables of the binomial distribution.[36] The data in Table

Table 60–8 Comparison of Some Operating Characteristics of Several Sampling Plans Relevant to Examination for Pathogens

	For Lots with p = 0.10 (ie, 10% Units Contaminated)			*For Lots with P = 0.02 (ie, 2% Units Contaminated)*		
Sampling Plan	*P_a**	*P_r†*	*% of Lots Likely To Be Rejected*	*P_a**	*P_r†*	*% of Lots Likely To Be Rejected*
n = 60, *c* = 0	0.002	0.998	99.8	0.30	0.70	70
n = 10, *c* = 0	0.35	0.65	65	0.82	0.16	16
n = 5, *c* = 0	0.59	0.41	41	0.90	0.10	10
n = 3, *c* = 0	0.73	0.27	27	0.94	0.06	6
n = 5, *c* = 1	0.92	0.08	8	0.996	0.004	0.4

* P_a means probability of acceptance of lot
† P_r means probability of rejection of lot

Source: Adapted with permission from ICMSF, *Micro-organisms in Foods. Vol. 2. Sampling for Microbiological Analysis: Principles and Specific Applications*, 2nd ed., © 1986, University of Toronto Press.

Table 60–9 Operating Characteristics for Various Single- and Double-Sampling Plans at Different Acceptance Quality Levels (AQL) Showing Incidence of Defective Units Likely To Be Accepted with a 95% Probability ($P_a = 0.95$) and Rejected with a 90% Probability ($P_r = 0.90$)

					Number of Defective Units (c)		% Defective Units	
Sampling Scheme	*Size of Lot (units)*	*AQL (%)*	*Sample Set*	*Number of Sample Units To Test (n)*	*Accept*	*Reject*	*Accepted at $P_a = 0.95$*	*Rejected at $P_r = 0.90$*
Single	2,000	1.0	Single	50	1	2	0.71	7.56
		4.0	Single	50	5	6	5.34	17.8
		10.0	Single	50	10	11	12.9	29.1
Double	2,000	1.0	First	32	0	2	1.3	10.1
			Second	32	0	1	*	*
			Cumulative	64	1	2	0.7	7.6
		4.0	First	32	2	5	4.39	19.7
			Second	32	2	3	*	*
			Cumulative	64	6	7	5.34	17.8
		10.0	First	32	5	9	13.1	34.1
			Second	32	4	5	*	*
			Cumulative	64	12	13	12.9	29.1

* This value is not relevant because if the second set of samples is analyzed, the result must be calculated on the cumulative sample size (ie, first + second samples)

Source: Data from Sampling Procedures for Inspection by Attributes: Part 1. Specification for Sampling Plans Indexed by Acceptable Quality Level (AQL) for Lot-by-lot Inspection, BS6001: Part 1: 1972, amended April 1986, © 1986, British Standards Institute.

60–10 illustrate the effect of marginal defectives on the probability of acceptance of a lot having various proportions of defective and marginal defective units for sampling plans with $n = 10$ and $c_m = 0$ or $c_m = 2$. The data demonstrate that with a sampling plan of $c_m = 0$, the probability of accepting a lot (P_a) with 2% defective and 20% marginal defective units is 0.09, whereas for a plan with $c_m = 2$, the P_a is 0.55; similarly, if the lot contains 10% defective and 20% marginal defective units, $P_a = 0.04$ and 0.24 for $c_m = 0$ and $c_m = 2$, respectively.

The probabilities for acceptance based on a range of three-class plans for various levels of defective and marginally defective product are shown in Table 60–11. In the same way that an OC curve can be derived for a two-class plan, based on the expected incidence of defective product, an OC curve for a three-class plan can be derived based on the incidence of defective and marginally defective (or marginally acceptable) product. The OC curve for such a plan (Figure 60–8) is described by the geometric surface of a three-dimensional plot because for any value of defective units there will be a range of values for marginally defective samples.

60.2.5 The Statistical Basis of Variables Sampling Plans

A major disadvantage of any Attribute sampling scheme applied to a measurable parameter is that it makes no assumption about, nor allowance for, the distribution of measurable data associated with a lot. In the case of products for which defectives can be determined by inspection, this is of no consequence; however when results are based on the interpretation of quantitative data against predetermined criteria, which define whether the test data are or are not acceptable, there is an excessive risk of unfairly

Table 60–10 Probability of Acceptance for a Three-Class Plan with $n = 10$ and $c_M = 0$ According to the Proportions of Defective (q) and Marginally Defective (p_1) Units in a Lot and the Stringency of the Sampling Plan (Dependent on the Value of c_m)

Proportion of Marginally Acceptable Units	Probability for Acceptance (P_a) with Proportion of Defective Units (q) =							
	0.00	0.02	0.05	0.10	0.20	0.30	0.40	0.50
$n = 10$, $c_m = 2$								
0.50	0.05	0.04	0.03	0.02	0.01	<		
0.40	0.17	0.14	0.10	0.06	0.02	<		
0.30	0.38	0.31	0.23	0.13	0.04	0.01	<	
0.20	0.68	0.55	0.41	0.24	0.07	0.02	<	
0.10	0.93	0.76	0.56	0.32	0.10	0.03	0.01	<
0.00	1.00	0.82	0.60	0.35	0.11	0.03	0.01	<
$n = 10$, $c_m = 0$								
0.50	<	<						
0.40	0.01	<	<	<				
0.30	0.03	0.02	0.02	0.01	<			
0.20	0.11	0.09	0.07	0.04	0.01	<		
0.10	0.35	0.29	0.21	0.12	0.04	0.01	<	
0.00	1.00	0.82	0.60	0.35	0.11	0.03	0.01	<

< means $P_a < 0.01$

Source: Adapted with permission from B. Jarvis, Statistical Aspects of the Microbiological Analysis of Food. *Progress in Industrial Microbiology*, Vol. 21, p. 74, © 1989, Elsevier Applied Science.

rejecting otherwise acceptable lots. Indeed, Clark[11] showed that use of a three-class sampling plan with $n = 5$, $c = 1$ provides a 1 in 12 chance of rejecting a lot with 0% defectives and 10% marginal defectives—such a risk is unacceptable to a manufacturer and can result in wastage of much valuable foodstuff. Further examples are given in Table 60–11.

Analysis of much chemical and physical data is done on the basis of statistical analysis of the data and comparison of the mean and variance of the data with prescribed criteria. Relatively little attention has been paid to the application of Variables sampling schemes in applied microbiology. However, Kilsby and colleagues[30,32] showed that variables sampling can be used with quantitative microbiological data such as those derived from colony count procedures. It has been claimed[20] that use of variables sampling permits "better decision processes" with "economic benefits." Application of a Variables scheme does require statistical analysis of the data to determine the variance of the counts; and the data must conform to, or be capable of transformation to, a normal distribution. Because most colony count data conform approximately to a log-normal distribution, the raw data can be transformed simply by taking logarithms prior to statistical analysis.

As with Attributes sampling, it is necessary to define a critical level of microbial count (C) deemed to be the boundary between acceptable and unacceptable material; and to define a permissible percentage, d, of units in a lot that may be allowed to exceed this critical level to decide whether or not the lot is satisfactory. For data that conform to a normal distribution, the proportion (K) of values exceeding C depends solely on the population distribution and is given by:

$$(C - \mu) / \sigma = K$$

where μ = population mean and σ = population standard deviation. By reference to tables of the standardized normal deviate (eg, Table 1 of Pearson and Hartley[36]) the percentage of values beyond C can be derived for any value of K. For example, 20% of values beyond C would correspond to a value for K of 0.84, while 5% beyond C would be equivalent to K = 1.645. Conversely, the critical value of K (K_d) corresponding to d may be determined: for example, if d = 5%, $K_d = (C-\mu)/\sigma \leq 1.645$. Low values for K imply high percentages beyond C so that rejection may be warranted.

Because in most analytical situations neither μ nor σ will be known, they may be estimated from

Table 60–11 Probabilities of Acceptance (P_a) for Three-Class Attributes Sampling Plans When the Lot Examined Contains 5%, 20%, or 50% of Marginally Defective Sample Units with Various Proportions Exceeding M and Different Sizes (n) of Sample Units. The plans assume that marginally defective sample units can be accepted at various levels (ie, $c_m =$ 0, 1, 2, or 3) and that no defective units (ie, > M) are acceptable (c_M).

		Probability of Acceptance (P_a) When							
Sampling Plan		5% > m*		20% >m			50% >m		
n	c	0% > M†	5% >M	0% > M	10% > M	20% > M	0% > M	25% > M	50% > M
5	0	0.77	0.59	0.33	0.19	0.11	0.03	0.01	<††
5	1	0.97	0.75	0.74	0.44	0.29	0.19	0.05	0.01
5	2	1.00	0.76	0.94	0.55	0.31	0.50	0.12	0.02
5	3	1.00	0.77	0.99	0.58	0.33	0.81	0.19	0.03
10	0	0.60	0.36	0.11	0.04	0.01	<	<	<
10	1	0.91	0.55	0.38	0.13	0.04	0.01	<	<
10	2	0.99	0.59	0.68	0.24	0.07	0.05	<	<
10	3	1.00	0.60	0.88	0.31	0.10	0.17	<	<
20	0	0.36	0.13	0.01	<	<	<	<	<

* 5% > m means 5% marginally defective sample units, etc.

† 0% > M means 0% defective units, etc.

†† < means P_a less than 0.005

Source: Adapted with permission from ICMSF, *Micro-organisms in Foods. Vol. 2. Sampling for Microbiological Analysis: Principles and Specific Applications*, 2nd ed., © 1986, University of Toronto Press.

the sample data by the statistics $\bar{x}$ and s. Hence, an estimate of K ($\hat{K}$) is given by:

$$\hat{K} = (C - \bar{x})/s$$

The estimate $\hat{K}$ is as likely to exceed K_d as it is to fall below it. Therefore, an allowance for the lack of precision of $\hat{K}$ is derived by determining a value k, such that the probability of $\hat{K} < k$ is at least P when $\hat{K} < K_d$, and P is the desired lowest probability for rejection. Values for k may be calculated for specific circumstances[7,32] or may be obtained from standard tables (eg, Bowker and Goode, Table K).[8] For low values of n, the values of k derived by mathematical approximation[32] were found to be insufficiently precise, and revised values (Table 60–12) based on the central limit theory have been published.[32]

For an upper critical level of C, the acceptance criterion is given by: $\bar{x} + k_M s \leq C$ and the rejection criterion by: $\bar{x} + k_M s > C$. This is illustrated diagrammatically in Figure 60–9. Any value lying above the line for K is rejected, whereas a value lying below the line is accepted. The Variables sampling scheme can be applied to microbiological food control through the establishment of a safety critical value (C_M) and a Good Manufacturing Practice (GMP) critical value (C_m), in a manner analogous to the three-class Attributes plan.

The Variables scheme is used by calculating the mean and standard deviation of the log colony counts (log cfu) and determining values for k from Table 60–12A (safety/quality) or Table 60–12B (GMP limit). Suppose that two sets each of five sample units have been analyzed from two different lots of a product and that the absolute upper critical limit (C_M) is 7.0 (log cfu) and the GMP limit (C_m) is 6.0 (log cfu). Suppose also that the producer wishes to reject, with a 95% probability, any lot where the proportion (d) exceeding C_M is 5% or greater and to accept with a probability of 90% only those lots where the proportion exceeding C_m is less than 10%. Suppose that the mean ($\bar{x}$) log transformed colony counts of samples from lots A and B are 5.20 and 5.90 with standard deviation (s) of 0.25 and 0.24, respectively.

Then from Table 60–12A, the value of k_M is 4.2 and, from Table 60–12B, that of k_m is 0.68. On safety criteria, if $\bar{x} + 4.2\,s$ exceeds a value of 7.0 (ie, C_M), the lot must be rejected. For lot A, the derived value is given by $5.20 + (4.2 \times 0.25) = 5.20 + 1.05 = 6.25 < 7.0$;

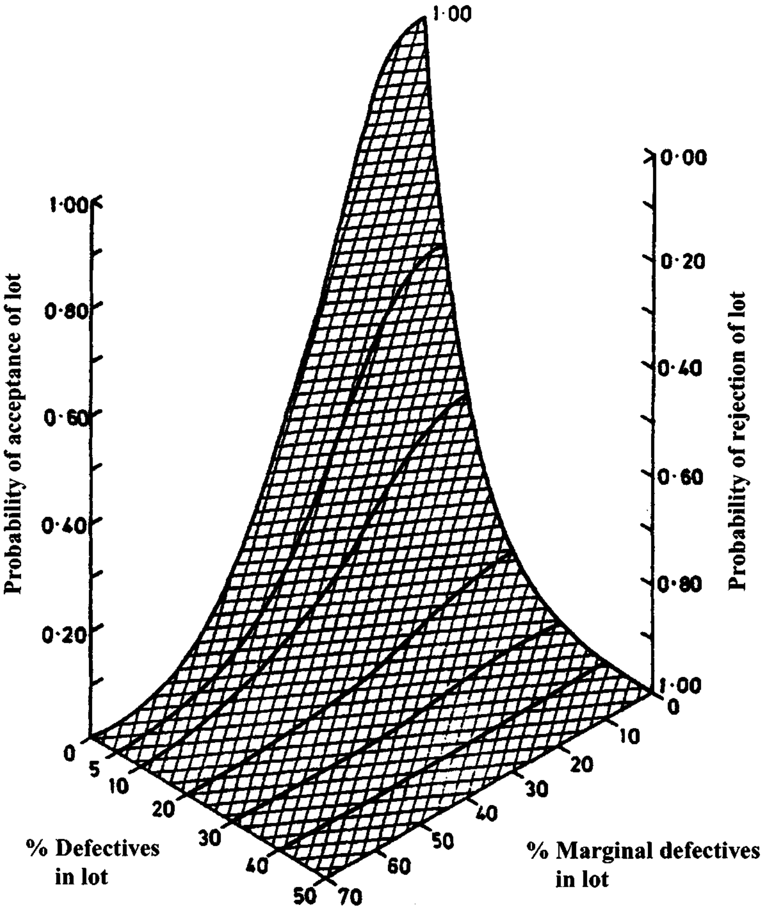

Figure 60–8 Operating Characteristics (OC) Curve Surface for a Three-class Sampling Plan with $n = 10$ and $c = 2$. *Source:* Reprinted with permission from B. Jarvis, Statistical Aspects of the Microbiological Analysis of Food, *Progress in Industrial Microbiology,* Vol. 21, p. 79, © 1989, Elsevier Applied Science.

hence, the lot is not rejected on the safety criteria. Similarly the derived value for lot B is given by 5.90 + (4.2 × 0.24) = 5.90 + 1.01 = 6.91 < 7.00 and the lot is (just) not rejected. From Table 60–12B, the GMP criteria state that $\bar{x} + 0.68\,s$ must not exceed a value of 6.0 (C_m) if the lot is to be accepted. For lot A, the derived value is given by 5.20 + (0.68 × 0.25) = 5.20 + 0.17 = 5.37 < 6.0 and the lot is accepted; but for Lot B, the derived value is given by 5.90 + (0.68 × 0.24) = 5.90 + 0.16 = 6.06 > 6.0 and the lot is not accepted. Hence, lot A would be accepted on both safety and GMP criteria; although lot B would not be accepted on the GMP criteria, it would be just acceptable on the safety criteria.

60.2.6 Variables or Attributes Sampling Plans?

There is no alternative option but to use a two-class Attributes sampling plan for assessment of product for the presence or absence of a specific group of organisms (eg, salmonellae). However, in any circumstance where quantitative data are obtained, the option exists to use either a three-class Attributes plan or a Variables sampling scheme. Is either better than the other?

In the case of the three-class plan, the results are attributed as being acceptable, marginally acceptable, or unacceptable by reference to the limits established for the plan (ie, *m* or *M*) and it is immedi-

Table 60–12 Values of *k* for Use in Setting Specifications for Variables Sampling Schemes Calculated Using the Noncentral *t* Distribution

(A) ***Safety/quality specification—reject lot if $\bar{x} + k_M s > C_M$, where $\bar{x}$ = mean log colony count, s = standard deviation of the log colony count, k_M = a constant, and C_M = safety limit***

Probability of Rejection (P_r)	Proportion (p_d) Exceeding C_M	k_M Values for the Following Number of Replicate (n) Sample Units							
		3	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0.95	0.05	7.7	5.1	4.2	3.7	3.4	3.2	3.0	2.9
	0.1	6.2	4.2	3.4	3.0	2.8	2.6	2.4	2.4
	0.3	3.3	2.3	1.9	1.6	1.5	1.4	1.3	1.3
	0.5	1.7	1.2	0.95	0.82	0.73	0.67	0.62	0.58
0.90	0.1	4.3	3.2	2.7	2.5	2.3	2.2	2.1	2.1
	0.25	2.6	2.0	1.7	1.5	1.4	1.4	1.3	1.3

(B) ***GMP limit—accept if $\bar{x} + k_m s < C_m$, where $\bar{x}$ = mean log colony count, s = standard deviation of the log colony count, k_m = a constant, and C_m = GMP limit***

Probability of Acceptance (P_r)	Proportion (p_d) Exceeding C_M	k_M Values for the Following Number of Replicate (n) Sample Units							
		3	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0.95	0.10	0.33	0.44	0.52	0.57	0.62	0.66	0.69	0.71
	0.20	–0.13	0.02	0.11	0.17	0.22	0.26	0.29	0.32
	0.30	–0.58	–0.36	–0.24	–0.16	–0.10	–0.06	–0.02	0.00
0.90	0.05	0.84	0.92	0.98	1.03	1.07	1.10	1.12	1.14
	0.10	0.53	0.62	0.68	0.72	0.75	0.78	0.81	0.83
	0.20	0.11	0.21	0.27	0.32	0.35	0.38	0.41	0.43
	0.30	–0.26	–0.13	–0.05	0.01	0.04	0.07	0.10	0.12
	0.40	–0.65	–0.46	–0.36	–0.30	–0.25	–0.21	–0.17	–0.16
	0.50	–1.09	–0.82	–0.69	–0.60	–0.54	–0.50	–0.47	–0.44
0.75	0.01	1.87	1.90	1.92	1.94	1.96	1.98	2.00	2.01
	0.05	1.25	1.28	1.31	1.33	1.34	1.36	1.37	1.38
	0.10	0.91	0.94	0.97	0.99	1.01	1.02	1.03	1.04
	0.25	0.31	0.35	0.38	0.41	0.42	0.44	0.45	0.46
	0.50	–0.47	–0.38	–0.33	–0.30	–0.27	–0.25	–0.24	–0.22

Source: Adapted with permission from B. Jarvis, Statistical Aspects of the Microbiological Analysis of Food, *Progress in Industrial Microbiology*, Vol. 21, p. 82, © 1989, Elsevier Applied Science with data from S. Malcolm, A Note on the Use of the Non-central t Distribution in Setting Numerical Microbiological Specifications for Foods, *Journal of Applied Bacteriology*, Vol. 57, pp. 175–177, © 1984, Blackwell Science, Ltd.

ately obvious whether any count exceeds these limits. However, the distribution of organisms can affect the proportion of values likely to lie between *m* and *M* or to exceed M, such that the Attributes sampling scheme fails to give a constant probability of rejection as the proportion exceeding *m* or *M* increases (Table 60–13).[32]

The use of a Variables sampling plan is predicated on the supposition that the data, after transformation, will conform to a normal distribution. It is pos-

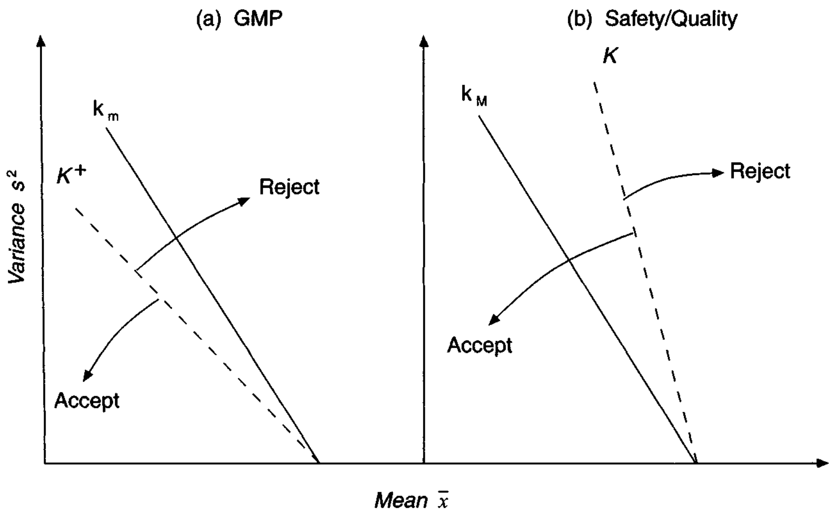

Figure 60–9 The Effect of the Standard Deviation in Microbial Counts in a Lot on the Response of a Standard Variables Scheme.[31] The lines k_m and k_M are derived from tabulated data (eg, Table 60–12) according to the number of replicate sample units allowed to exceed defined limits (C_m and C_M). The lines allow a fixed proportion of samples to exceed the limit and determine the proportion of defective product in the lot that will be rejected.

sible to check for normality using appropriate statistical techniques when the number of sample units tested is large (>100) but such tests cannot be applied if, as in most microbiological testing, the number of sampling units is small (often five or fewer replicate tests are carried out). If the data do not conform accurately to a normal distribution, then the use of Variables sampling plans may lead to wrong decisions resulting in excessive rejection or acceptance of a lot of product. Figure 60–10 illustrates a situation with two sets of data (A and B), each log-normally distributed and then the combined distribution, obtained by blending equal parts of the raw material (Figures 60–10A and 60–10B) and a ratio of 1 part A to 4 parts B (Figure 60–10B). Such an event could occur in industrial production where different batches of an ingredient may be used in the preparation of a single batch of food product; or in importation testing where more than one lot of product may be included in a single consignment. It is clear from Figure 60–10B, that when equal quantities of the ingredients are blended, the combined distribution of counts is no longer normally distributed, but the lack of normality is more difficult to observe when 1 part of A is blended with 4 parts of B because, in this case, only a small degree of skew occurs. To test for nonnormality requires application of an effective test, but the only reliable statistical procedures to make this test require a large number of data points to be available.

60.3 CHOICE OF SAMPLING PLANS

Effective sampling is based on the presumption that all sample units are drawn randomly from a lot, so that the quality of any one sample unit is typical of, and neither better nor worse than, the population from which it is drawn. It is therefore important to draw sample units to conform with the requirements of a sampling plan without introducing any bias in the selection of samples. While there is no guarantee that the quality of sample units drawn randomly from a lot will be typical of that lot, at least no bias will have been introduced in the choice of sample units.

The simplest method is to allocate a number to each unit in the lot and then to determine random numbers from a random number table[19] or generated by computer. In practice, this approach is rarely possible when a lot consists of large quantities of product produced under modern manufacturing conditions or held in the hold of a ship. It is easier to use a random number sampling scheme during manufacture when sample units can be withdrawn throughout the production process, but even then it may be almost impossible to ensure that a specific sample is

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Table 60–13 Comparison of an Attributes and a Variables Sampling Scheme.

Probability of rejecting a batch using a three-class attributes plan of $n = 5$, $c = 3$ or a variables plan with 5 sample units (n), $p = 50\%$ and $P = 0.99$.

	Proportion of Lot between *m* and *M**	P_r with Proportion of Lot Exceeding C_M† or M†=				
		0.10	*0.20*	*0.30*	*0.40*	*0.50*
Variables plan	—	0.81	0.90	0.95	0.98	0.99
Attributes plan	0.1	0.41	0.67	0.83	0.92	0.97
	0.3	0.48	0.70	0.85	0.94	1.00
	0.5	0.68	0.80	0.93	0.98	1.00
	0.7	0.94	0.96	1.00	1.00	1.00

* Proportion of marginally defective units

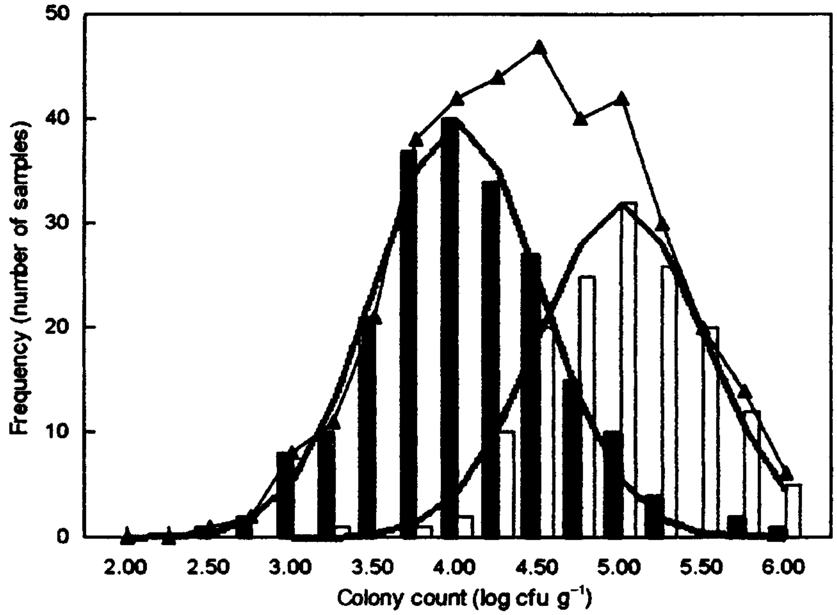
† C_M is the upper limit for the operation of a variables sampling plan for food safety (see also Table 60–12); M is the upper limit defined in ICMSF sampling plans[24]

Source: Adapted with permission from D.C. Kilsby and A.C. Baird-Parker, Sampling Programmes for the Microbiological Analysis of Food, in *Food Microbiology: Advances and Prospects*, T.A. Roberts and F.A. Skinner, eds., pp. 309–315, © 1983, Academic Press, Inc.

taken rather than an adjacent sample. Bulk liquid and granular products can be sampled using either a discontinuous or continuous sampling system (qv) or on a random time basis. However, once a product has been put into its primary packaging, then multipacked into cases and palletized, the application of totally unbiased random sampling procedures becomes almost impossible in practice. Several approaches to modifying the ideal scheme are described below. One approach is to deliberately stratify the lot to permit sample units to be withdrawn from each of a given number of different strata (eg, cases, pallets, containers, ship's holds). The objective is to provide an equal opportunity for sample units from different strata to be in the total strata.

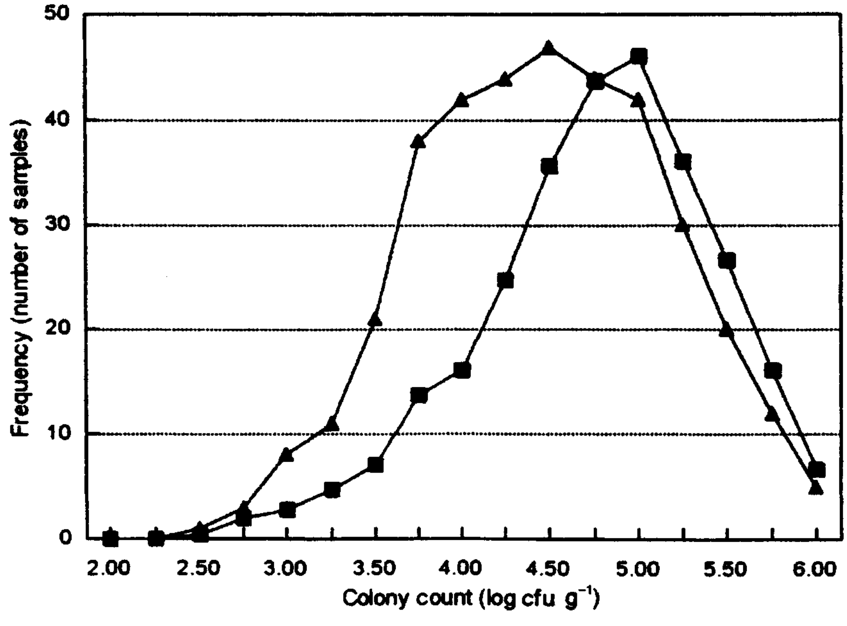
60.3.1 Multiple Sampling Schemes

These are primarily applied to materials packaged in definable unit portions. Each bulk unit (eg, a pallet of cases) is allocated a number and the requisite number of bulk sample units is drawn using random numbers. One or more individual sample units (eg, cases) is then drawn from each bulk unit, again using random numbers. In many circumstances, it may also be necessary to sample from the individual case, thus leading to sets of samples being drawn from each level of packaging (eg, pallets, cases, individual product units [eg, packages or bottles]). This is a practical approach to minimize bias when it is necessary to draw a number of random samples from a consignment consisting of a large single lot of product (eg, a day's production held in a warehouse). An example of a procedure for multiple sampling is given in Table 60–14, which illustrates the effects of the different sampling regimes on the probability of acceptance or rejection of a lot subjected to multiple sampling.[9] These data assume a lot consists of 1,400 sacks of product stacked on 70 pallets each of 20 sacks. Assuming that the AQL required is given by AQL = 4%, then using a single sampling plan based on a "normal" level of sampling (qv), it would be necessary to draw a total of 125 sample units, of which a maximum of 10 units could be accepted as being defective (ie, $n = 125$, $c = 10$). Such a scheme would accept 4.9% defectives at $P_a = 0.95$ and would reject 12.3% defectives at $P_r = 0.90$. However, for a similar AQL, a double-sampling scheme would require testing 5 randomly drawn sacks (out of 20) from each of 13 pallets, each selected randomly from the 70 pallets; 1 pallet could be accepted as a defective unit. Such a scheme would accept up to 2.8% defectives at $P_a = 0.95$ but would only reject at $P_r = 0.90$ if 37% of units were defective. Clearly, such a multiple-sampling scheme is less stringent than a



A

Data from ingredient A (■) ($\bar{x}$ = 4.04, s^2 = 0.29) and from ingredient B (☐) ($\bar{x}$ = 4.99, s^2 = 0.27) are each overlaid with the "normal variate" for their respective mean values; and the distribution curve resulting from combination of the materials in equal parts ($\bar{x}$ = 4.50, s^2 = 0.50) (▲----▲)



B

The distribution curves resulting from combination of the materials in the ratio 1 part ingredient A and 4 parts ingredient B ($\bar{x}$ = 4.80, s^2 = 0.44) (■---■) and equal parts of ingredients A & B ($\bar{x}$ = 4.50, s^2 = 0.50) (▲----▲)

Figure 60-10 Non-normality of Distribution Data Arising from Combination of Two Sources of Raw Material, Each Log-Normally Distributed and Having Similar Variance

single-sampling scheme in rejecting unsatisfactory materials but is often a pragmatic solution to the problem of drawing samples from large lots.

60.3.2 Stratified Sampling Schemes

When a consignment of product consists of several identifiable lots of product, it is essential to sample each lot within the consignment individually. The sample units drawn from each lot within the consignment should then be treated individually; results from the individual lots should be pooled only if evidence of homogeneity is obtained in the individual sets of results.

60.3.3 Sampling Frames

Sometimes it is not possible to sample a whole lot or consignment because of problems of accessibility. Such a situation might arise with stacks of boxes or sacks within a warehouse or a ship's hold. In such a situation, sample units are drawn, as described above, from those parts of the lot that are accessible (this is referred to as the "frame"). The results of the analysis apply only to the frame, not to the whole lot or consignment. However, if several frames are tested and the results indicate homogeneity, it is a reasonable presumption that the results on the frames tested may be indicative of those that might have been obtained by random sampling of the entire consignment or lot.

60.3.4 Deliberate Sample Bias

One traditional approach to sampling in the food industry is to deliberately sample a production line at a time when a problem might be expected to occur. For instance, if a pasteurized liquid product is being "clean" filled, it is often normal practice to test the first bottles filled after plant cleaning to provide evidence that the plant was effectively cleaned. While this may have some practical benefits in identifying inadequate cleaning of the packaging machinery, the data merely provide a retrospective verification that all was or was not satisfactory. The data apply only to those first sample units and cannot be related to the production lot as a whole. With modern rapid methods for checking plant hygiene (eg, analysis of adenosine triphosphate [ATP] residues), there is really little justification for such an approach to be used.

60.3.5 Normal, Tightened, and Reduced Sampling Schemes

Various published schemes for Attributes sampling by inspection or examination recommend the establishment of a protocol that varies the intensity of sampling according to the expected risk.[9] It is recommended, for instance, that under routine manufacturing or surveillance conditions, a sampling scheme should be operated using a *Normal* level of sampling selected according to the lot size and the

Table 60–14 Comparison of % Defectives Likely To Be Accepted with $P_a = 0.95$ and Rejected with $P_r = 0.90$ for a Single Sampling Plan or a 2-Stage Stratified Sampling Plan for Bulk Materials

					Approximate % Defectives	
Number of Units in Lot	*Sampling Stage*	*Number of Units To Test (n)*	*Number of Defectives Acceptable (c)*	*AQL (%)*	*Accepted at $P_a = 0.95$*	*Rejected at $P_r = 0.90$*
1,400 sacks	Single	125	10	4.0	4.9	12.3
70 pallets	First	13	1	4.0	2.8	26.8
20 sacks	Second	5	0	2.5	1.02	36.9
	Combined*	65	<5	4.0	2.8	36.9

*ie, 5 sacks from each of 13 pallets giving a total of 65 sacks to be sampled.

Source: Adapted with permission from B. Jarvis, Statistical Aspects of the Microbiological Analysis of Food, *Progress in Industrial Microbiology*, Vol. 21, p. 86, © 1989, Elsevier Applied Science.

required AQL. However, if the risk associated with a particular process, or a specific source of product, is considered to be low, then a *Reduced* level of sampling may be used until evidence suggests a need to reintroduce *Normal* sampling. Likewise, if evidence is found for an increased hazard, then a *Tightened* sampling scheme should be used. Specific rules have been established to enable a decision to be made to move from one category to another (eg, *Reduced* to *Tightened* sampling).[9]

Table 60–15 illustrates a set of Reduced, Normal, and Tightened sampling schemes. The table shows that the number of:

- sample units to be examined increases with increasing lot size (see also Table 60–6)
- sample units required for a Reduced sampling protocol is less than for Normal or Tightened sampling
- acceptable defective units varies according to the plan, being fewer for the tightened scheme than for the normal scheme.

A lot size of 3,201 to 10,000 units requires the examination of 80 sample units for both Normal and Tightened inspection but only 32 sample units for Reduced sampling. For Normal inspection, the lot would be accepted with an AQL of 4%, provided not more than 7 samples were found to be defective (ie, $n = 80$, $c = 7$), but for the Tightened inspection, the lot would be accepted only if not more than 5 sample units were defective (ie, $n = 80$, $c = 5$). By contrast, the Reduced sampling scheme accepts not more than 3 defectives from the 32 sample units examined (ie, $n = 32$, $c = 3$) and rejects the lot if 6 defectives are found. In this instance, the test would need to be repeated if more than 3 but fewer than 6 defective sample units were found.

Schemes of this sort are very useful when the level of sampling can be changed according to the perceived need. For instance, it is possible to identify a production line as operating under green, amber, or red status according to recent experience of plant hygiene, or final product quality. Clearly, it is necessary to adopt rules to change from one state to another according to the perceived trends in contamination.

A similar approach was taken by ICMSF[24] in its recommended sampling plans for a variety of foods according to the perceived hazard and intended use. Table 60–4 illustrates the ICMSF recommended sampling plans for salmonellae in dried milk where the plan to be chosen is dependent on whether the expected handling, storage, and use of the product will reduce, cause no change in, or increase the level

Table 60–15 Single Sampling Schemes for Reduced, Normal, and Tightened Procedures for a Lot Size of 5,000 Units Showing Number of Defective Sample Units That Can Be Accepted (c) and the Approximate Incidence of Defective Units That Would Be Accepted with $P_a = 0.95$ and Rejected with $P_r = 0.90$

			Number of Defective Units		*Approximate % Defectives*	
AQL (%)	*Sampling Scheme*	*Number of Sample Units To Test (n)*	*Accept with (c)*	*Reject with*	*Accepted at $P_a = 0.95$*	*Rejected at $P_r = 0.90$*
1.0	Normal	80	2	3	1.0	6.5
	Tightened	80	1	2	0.4	4.8
	Reduced	32	1	3	0.7	7.6
4.0	Normal	80	7	8	5.1	14.2
	Tightened	80	5	6	3.3	11.3
	Reduced	32	3	6	4.4	19.7
10.0	Normal	80	14	15	12.0	24.2
	Tightened	80	12	13	9.9	21.4
	Reduced	32	7	10	13.1	34.1

Source: Data from Sampling Procedures for Inspection by Attributes: Part 1. Specification for Sampling Plans Indexed by Acceptable Quality Level (AQL) for Lot-by-Lot Inspection, BS6001: Part 1: 1972, amended April 1986, © 1986, British Standards Institute.

of concern. For use by a high-risk population, the recommended sampling plan requires testing of 60 sample units if the hazard is likely to increase (eg, as a result of holding the reconstituted product under unacceptable conditions before consumption). Only 15 sample units need to be tested if the level of concern is reduced (eg, by consumption immediately after reconstitution). In all cases, the sampling plans require rejection of the lot if salmonellae are detected in any sample unit.

60.4 STATISTICAL PROCESS CONTROL CHARTS

Traditionally, microbiological counts of viable aerobic organisms and/or specific groups of organisms (eg, coliforms or Enterobacteriaceae) or tests to detect the presence or absence of specific pathogens (eg, salmonellae) have been used in microbiological quality control procedures. The objective is to establish retrospectively whether an ingredient, a partly processed product, or a final product conforms with a predetermined specification. Products not conforming are considered to be outside specification, which might lead to product destruction, recall from the marketplace, or other consequence. Often the specifications are based on intuitive understanding of the significance of certain levels of microorganisms but are not necessarily related specifically to any assessment of producer's or consumer's risk. Often microbiological procedures used in industry are not based on properly evaluated microbiological criteria; therefore, decisions may often be based on analysis of a single sample unit, sometimes even without any replication of the microbiological test. The introduction of recommended microbiological criteria[24] has been helpful in increasing understanding of the importance of using effective sampling plans. Nonetheless, even today much microbiological testing in industry is carried out using inadequate sampling plans.

An important role for application of microbiological criteria is in verification, but not monitoring, of critical control points (CCPs) within an HACCP system. Microbiological criteria *cannot* be used to monitor CCPs because, by definition, monitoring implies a rapid evaluation that traditional microbiological test procedures cannot provide. However, traditional methods can provide valuable retrospective data to verify the correctness of decisions based on the automated monitoring of chemical and physical criteria or alternative biological procedures, such as analysis of plant hygiene swabs for ATP.

Unfortunately, having spent considerable amounts of money on technician time and laboratory media to generate microbiological data for quality control purposes, the data are often examined in a cursory manner and then filed for posterity. How much better it would be if the data were analyzed further to provide evidence of trends that may be occurring. Such trends can be helpful also in the laboratory to establish whether colony count or other laboratory procedures are in control (see Section 60.5.3).

60.4.1 Statistical Process Control (SPC)

Increasingly, industry is adopting automated monitoring and control of manufacturing processes; regrettably, it is not yet possible for microbiological procedures to be incorporated in a timely manner in such systems of SPC. However, critical control parameters such as are used in HACCP (eg, temperature, pressure, pH, viscosity) and some chemical parameters can be monitored in this manner. The basis of SPC is the continuous analysis of process parameters so that the data can be assessed against predetermined criteria by a process control computer system. Such criteria are determined by surveying a process operating GMP over time in order to generate parameters for the expected mean, median, range, and/or variance of the data analyzed. Data from analyses of continuous variables (eg, temperature, pressure) generally conform to a normal statistical distribution where 50% of the data lie on either side of the mean value although nonstandard distributions of data may be observed, which require transformation to "normalize" the data before analysis. Once the critical parameters have been determined, the control computer can be programmed to enable continuous monitoring of new process data against these criteria and to identify when a system starts to go out of control. Indeed, in some industries, the control computer can adjust process parameters to remedy any trend that is detected outside predetermined limits. More information on SPC can be found in standard texts.[6,35,41]

60.4.2 Statistical Control Charts

The basis of SPC is essentially the use of statistical control charts such as the Shewart and Cusum charts,[6] which have been used by industrial statisticians for many years. The objective is to use process control data to monitor trends against predetermined criteria. Figure 60–11 illustrates the elements of a Shewart control chart having as a baseline a standard variable (eg, mean, standard deviation, proportion) together with upper and lower control limits. Such a chart could be used for colony count, MPN, multitube presence or absence test data, or hygiene swab ATP levels. The baseline (control limit [CL]) of the chart might be the process average ($\bar{x}$), the standard deviation (s), the range (R), or individual data. Above and, in most circumstances also, below the CL are two parallel lines: the upper and lower control limits (UCL; LCL). To establish a control chart, a minimum of 20 sets, each of at least 5 sample units, of process samples are analyzed. The average and range are calculated for each set of sample units and tested for compliance with normality by plotting a histogram either of the average values or of the values after normalization (eg, by taking log values). Assuming that the data conform with normality, the overall process average ($\bar{\bar{x}}$) and the overall process range ($\bar{R}$) are calculated. These values then become the CL (CL_x and CL_R) for $\bar{x}$ and R charts, respectively:

$$\mathbf{CL_x = \bar{\bar{x}} = \sum \bar{x} / n \quad CL_R = \bar{R} = \sum R / n}$$

where $\bar{x}$ = sample average, R = sample range, and n = number of sample sets.

The UCL and LCL values are derived from the process average ($\bar{\bar{x}}$) and range $\bar{R}$:

$$\mathbf{UCL_x = \bar{\bar{x}} + k_1\bar{R} \qquad UCL_R = k_3\bar{R}}$$

$$\mathbf{LCL_x = \bar{\bar{x}} - k_1\bar{R} \qquad LCL_R = k_2\bar{R}}$$

where the constants (k_1, k_2, and k_3), set at ± 3 s, are obtained from tables and are dependent on the sample size (n) (see Table 60–16). It should be noted that R is used to estimate the standard deviation, that different values of k_2 and k_3 are used for the lower and upper limits for range charts to reflect the different mode of calculation, and that no LCL_R is established for small numbers of sample units (n).

The interpretation of data on Shewart control charts is well defined: there are six recognized indicators of lack of control. These are:

1. a single point outside the UCL and LCL values
2. seven consecutive points falling on one side of the center line
3. two or more points in a row falling close to the UCL or LCL
4. ten of 11 points on one side of the center line
5. a trend of seven or more points in a row upward or downward
6. cycling of points above and below the target line.

Further information and illustrated examples can be found in Beauregard, Mikulak, and Olson.[6]

A Cusum chart (Figure 60–12) operates on a similar basis but, instead of plotting the actual data, the cumulative sum of the deviation between individual data values (or mean values) and a reference value is plotted on a rolling basis. Cusum charts do not normally have control limits but instead have upper and lower cumulative signal values. Cusum chart masks can be used as aids to assess statistically whether there has been a change in the process, but are not recommended by Montgomery.[34]

Table 60–17 presents data on microbiological counts determined on a manufactured food product over a period of several weeks. The reference values used are the target log mean count ($\bar{\bar{x}}$; expected to be 3.0 [ie, 1000 cfu g^{-1}]) with an upper log colony count limit of 3.5 (~3100 cfu g^{-1}). Figure 60–13A illustrates the plot of the individual mean log counts ($\bar{x}$) against time. Two small trends can be seen on this graph (from day 7 and again from day 15), but the data do not demonstrate lack of control as defined above until day 23 when two values exceed the upper Cusum control limit. A plot of the cumulative differences [$\Sigma(\bar{x}_i - \bar{\bar{x}})$] on a Cusum chart (Figure 60–13B) demonstrates a continual and significant upward trend from day 8 until day 23. This graph makes the development of an adverse trend much clearer than that seen in Figure 60–13A.

Similarly, Figures 60–14A and B show the use of charts to monitor trends in plant hygiene determined by measurement of ATP levels on swabs taken from the mandrel of a milk filling machine after cleaning.[22] Because these data were obtained from single samples ($n = 1$), traditional data analysis systems (eg, Shewart charts) are not suitable. Hayes and colleagues[22] presented their analysis after square root transformation to normalize the raw data on the assumption that the occurrence of ATP on production plant follows a Poisson distribution. However, there is no necessity for this transformation, because residual ATP levels will not necessarily reflect microbial contamination but rather will be indicative of ATP arising from both food residues and microbial cells. Data obtained by replicate analyses of ATP on process plant generally follow a log-normal rather than a Poisson distribution (Lindhardt, personal communication). The data, transformed to show the

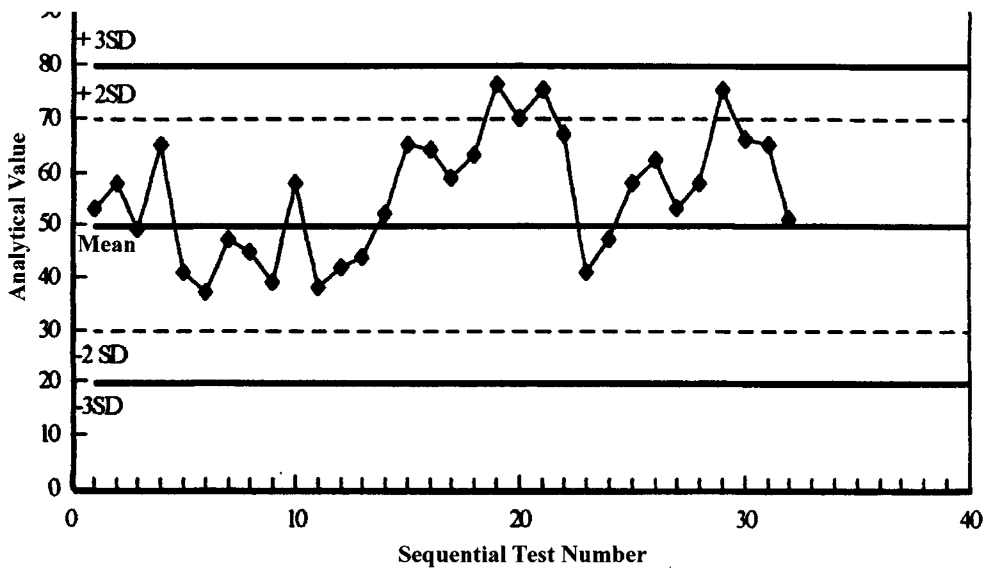


Figure 60–11 An Example of a Shewart Chart with Lines for the Process Mean (Control Limit), Warning (Upper and Lower Limits- - - - ± 2SD) and Action (Upper and Lower Control Limits— ± 3SD) levels. Superimposed on the chart are hypothetical data values.

deviation from the target value (assumed to be 50 relative light units [RLU], being a typical level for ATP on cleaned dairy plant) plotted against time (Figure 60–14A), show considerable day-to-day cycling of ATP levels—but no significant trends until day 73 when an unacceptable level of ATP (> 250 RLU) was determined. However, if the cumulative deviations in ATP levels are plotted as a Cusum chart (Figure 60–14B), significant trends can be seen from day 65, with other adverse trends having occurred also on days 23 to 30, 36 to 40, 46 to 48, and 84 to 90.

Loss of control is seen even more readily in Figure 60–14C, a single-sided Tabular Cusum plot.[29,34] The upper signal factor used (C_i^+) is derived mathematically as the greater value of zero or the calculated Cusum value from the equation:

$$C_i^+ = \max \left\| 0, x_i - (\mu_0 + K) + C_{i-1}^+ \right\|$$

where C_i^+ is the positive Tabular Cusum for the i^{th} data value (x_i); C_{i-1}^+ is the positive Tabular Cusum value for the previous data value (x_{i-1}); μ_0 is the target value and K is a reference value (sometimes

Table 60–16 Constants for Process Average and Range Control Charts

	Constant for		
		Range Chart	
Number of Sample Units (n)	*Average Value Chart Upper and Lower Limits UCL_x and LCL_x (k_1)*	*Lower Limits LCL_R (k_2)*	*Upper Limits UCL_R (k_3)*
2	1.880	—	3.267
3	1.023	—	2.574
4	0.729	—	2.282
5	0.577	—	2.114
10	0.285	0.223	1.777
20	0.180	0.415	1.585

Source: Data from M.R. Beauregard, R.J. Mikulak, and B.A. Olson, *A Practical Guide to Statistical Quality Improvement*, © 1992, Van Nostrand Reinhold.

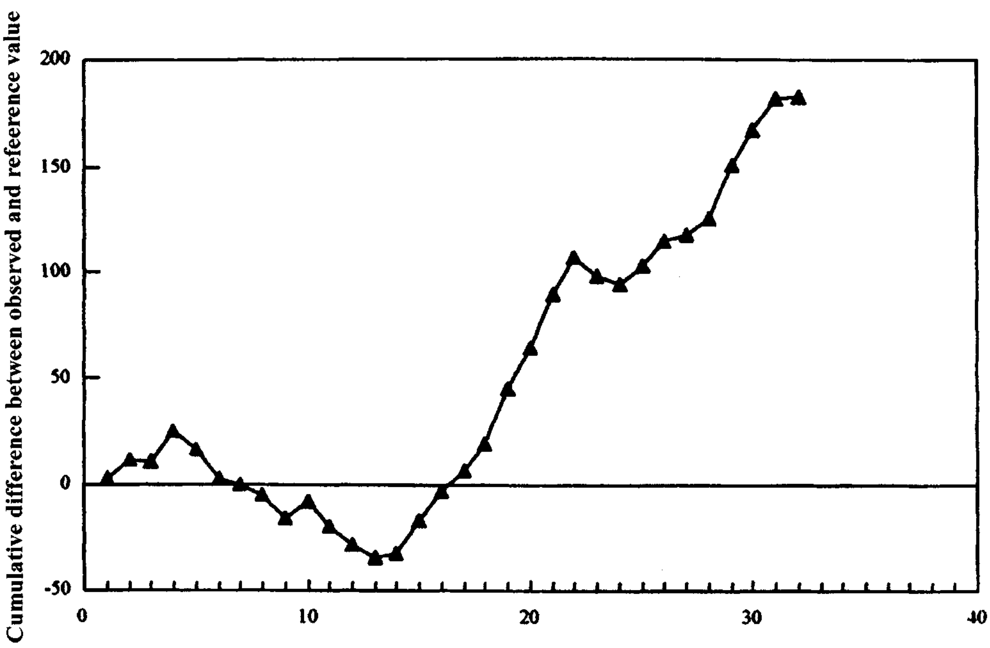


Figure 60–12 Cusum Chart. A plot of the cumulative sum of the differences between the actual data value and the process mean (target value) using data values from Figure 60–11.

called the allowance or the slack value). K is usually chosen as the value midway between the target (μ_0) and an "out of control limit value" (μ_1), that it is wished to detect as early as possible and is often 2, but may be up to 5, standard deviations above the target value. Thus $K = \frac{(\mu_1 - \mu_0)}{2}$. However, the Tabular Cusum can be applied only to data that conform to a normal distribution, so that microbial counts and ATP values must be normalized by taking logarithmic values of the data. Hence, as shown in Figure 60–14C, for which the target value (as log RLU) $\mu_0 = 1.7$, $s = \pm 0.2$ and the "out of control value" $(\mu_1) = \mu_0 + 2s = 1.7 + (2 \times 0.2) = 2.1$; then $K = (2.1 - 1.7)/2 = 1.9$. Figure 60–14C shows only those adverse trends in data that exceed the target value plus 1 standard deviation. Obviously, other target values and allowances could be used. For further information on preparation and use of Tabular Cusum, see Montgomery.[34]

The application of statistical analyses to routine data obtained in quality control analyses enables the information to be used positively to predict the development of adverse trends *before* a serious event occurs. Other types of control chart may also be useful in the assessment of data.[6,34,35,41]

60.4.3 Control of Microbiological Laboratory Data

Reference was made above (see Section 60.1.2) to the errors associated with microbiological testing. It is not appropriate in this chapter to consider the quality control of laboratory media, but it is important to draw attention to the need for quality assessment of laboratory media[40] (see also Chapter 62) and quality control of whole processes by the use of reference materials or foods. In considering the results of laboratory analyses, it is important to assess whether the laboratory analysis was in control by carrying out a simple statistical analysis of replicate data values.

Fisher and colleagues[18] were the first to demonstrate that abnormally large variations between replicate colony counts could be associated with factors causing contagious distribution of microorganisms and that subnormal variations (ie, regular distributions) are associated with culture media defects. As described above (see Section 60.1.1), a simple method to test whether a set of colony counts conforms with a Poisson distribution is to use Fisher's Index of Dispersion *(I)* test as a measure of the equality of variance and mean.

The statistical significance of the extent to which this ratio (*I*) departs from unity can be determined

Table 60–17 Production Control Chart Data for Standard Plate Counts on a Dairy Product

Day	Mean Colony Count$_i$ (Log cfu g^{-1}) ($\bar{x}_i$)	Difference from Target* ($\bar{x}_i - \bar{\bar{x}}$)	Cumulative Sum $\Sigma(\bar{x}_i - \bar{\bar{x}})$
1	2.6	–0.4	–0.4
2	2.9	–0.1	–0.5
3	3.4	0.4	–0.1
4	2.7	–0.3	–0.4
5	3.1	0.1	–0.3
6	2.9	–0.1	–0.4
7	2.6	–0.4	–0.8
8	2.8	–0.2	–1.0
9	3.3	0.3	–0.7
10	3.2	0.2	–0.5
11	3.5	0.5	0.0
12	2.9	–0.1	–0.1
13	3.1	0.1	0.0
14	3.3	0.3	0.3
15	2.8	–0.2	0.1
16	3.2	0.2	0.3
17	3.3	0.3	0.6
18	3.3	0.3	0.9
19	3.2	0.2	1.1
20	3.5	0.5	1.6
21	3.3	0.3	1.9
22	3.1	0.1	2.0
23	3.8	0.8	2.8
24	3.7	0.7	3.5
25	2.6	–0.4	3.1
26	2.4	–0.6	2.5
27	2.5	–0.5	2.0
28	2.8	–0.2	1.8

* target log mean count ($\bar{\bar{x}}$) = 3.0 cfu g^{-1}

from a table of χ^2 with (n –1) degrees of freedom or, more approximately, by comparison with a graph that relates the critical values for χ^2 against the number of replicate tests (Figure 60–5).

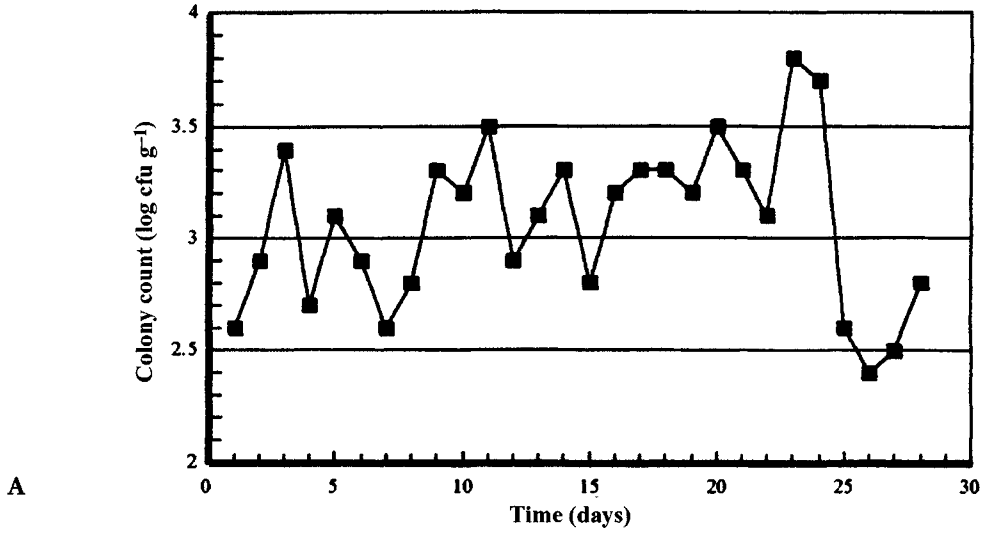
A χ^2 control chart can be used to test whether counts are in statistical control.[16] Because the critical value of the χ^2 test is dependent on the number of replicate colony counts, the procedure can be used only when at least duplicate counts are available at the same dilution level. An example of a χ^2 chart is shown in Figure 60–15. These data were derived from a series of colony counts of *Clostridium botulinum* and *C. perfringens*, using 20 replicate counts per test. Of the 32 sets of counts, one gave a χ^2 value marginally in excess of p = 0.025 and one fell below the value for p = 0.975. At the 5% level of significance, not more than 1 in 20 results should fall outside these limits if the hypothesis is correct that counts do not differ from a Poisson distribution. It may reasonably be concluded therefore that these counts essentially are in control. Obviously, these data are based on many more replicate counts than would normally be used in industrial practice, but even with duplicate counts useful information can be achieved by use of this procedure. A more detailed account of the application of these and other procedures has been published.[28]

60.5 SOME PRACTICAL ASPECTS OF SAMPLING AND ANALYSIS

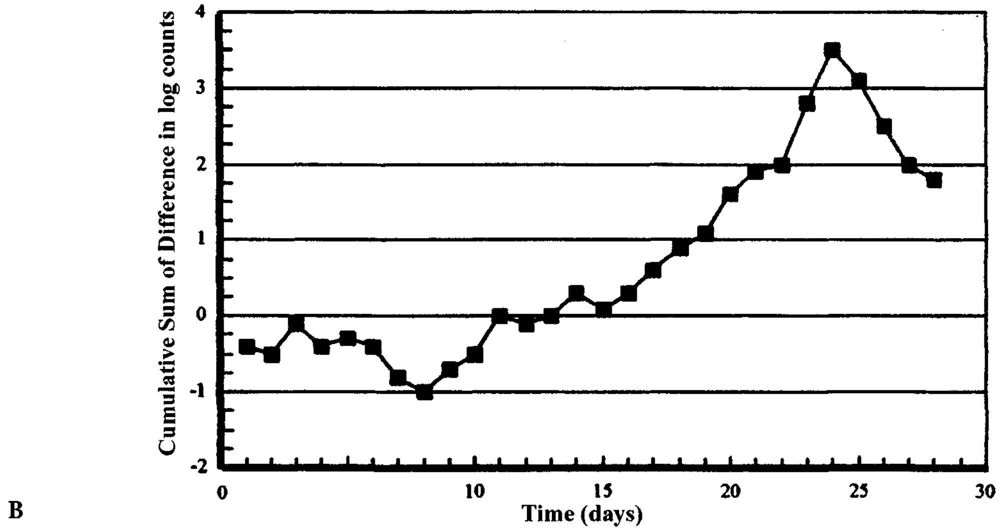
The primary objective of sampling is to enable decisions to be made concerning the acceptability of goods based on assessment of either Attribute or Variable characteristics determined by inspection, examination, or analysis. Specific objectives will vary according to the circumstances and may include assessing the efficiency of process plant cleaning and sanitation, ensuring that a lot of manufactured product conforms with agreed criteria, checking incoming raw or partially processed materials, and assessing imported goods at the port of delivery. The implications of using sampling plans with a limited number of sample units compared with the higher level of sampling recommended by national and international standards organizations have been discussed above in relation to the degree of confidence in decisions based on the laboratory data. Of considerable importance is the recognition that the reproducibility and repeatability of laboratory data for microbiological purposes is inherently low due to the impact of microbial distribution on the results obtained. What can be done to improve reproducibility and repeatability in the laboratory?

60.5.1 Size and Comminution of Primary Samples

The distribution of microorganisms in food normally follows either a Poisson (random) or, more usually, a negative binomial (contagious) distribution that is approximated by a log-normal distribution.[28,30,31] The extent of the analytical variance of data obtained on replicate sample units affects the number of samples that need to be drawn to have a reasonable degree of confidence in the results (Table 60–18). If, for instance, the variance (s^2) of the distribution of microorganisms in a lot is 0.04 (s = ± 0.2 log units), it would be necessary to examine 60 replicate sample units to be 95% sure that the mean log count is within 90% of the true average count of microor-



Plot of mean log colony count ($\bar{x}_i$) against time



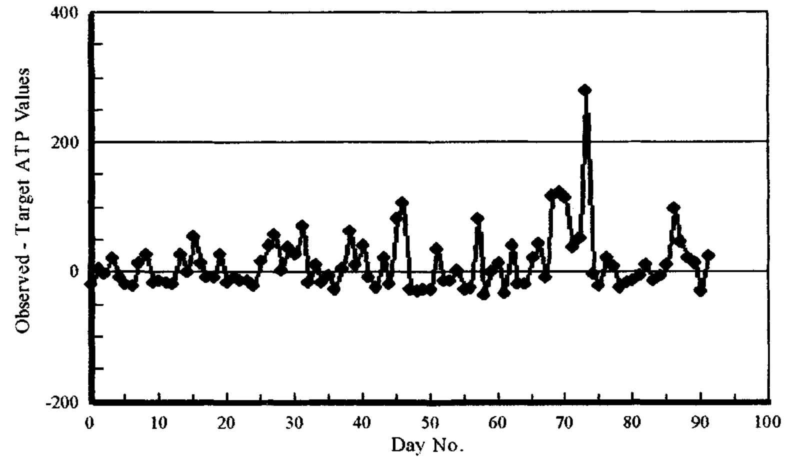
Plot of the cumulative differences between mean log colony count ($\bar{x}_i$) and a target value ($\bar{\bar{x}}$) of 3.0 (log cfu g^{-1})

Figure 60–13 Shewart and Cusum Charts for Colony Count Data Obtained from a Dairy Product (Using the Data in Table 60–17)

ganisms in that lot. However, if $s^2 = 0.25$ ($s = \pm 0.5$ log units), it would be necessary to examine 699 samples to have the same degree of confidence, although a lesser degree of confidence (ie, 90% sure of being within 50% of the true average count) could be obtained by testing 12 replicate sample units. Some values for microbial variance in food materials are shown in Table 60–19. Clearly, the higher the level of variance, the lower the degree of confidence that can be placed on the results.

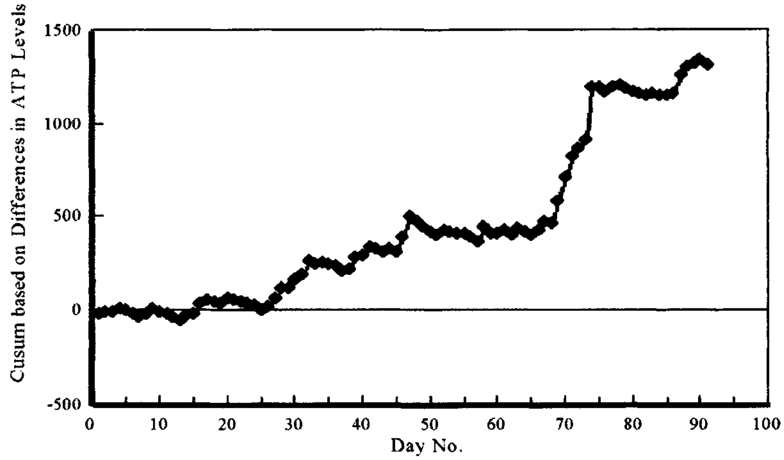
The effect of microbial variance in a product can be minimized by increasing the analytical sample unit size. Table 60–19 illustrates the effects of increasing the size of the analytical sample unit on the variance of microbial counts obtained on replicate sample units taken from a frozen block of meat. Increasing the size of the analytical sample from 20g to 500g reduced the variance of counts from 0.274 to 0.026. Similarly, comminution of the meat after thawing by mincing and bowl chopping also reduced

A



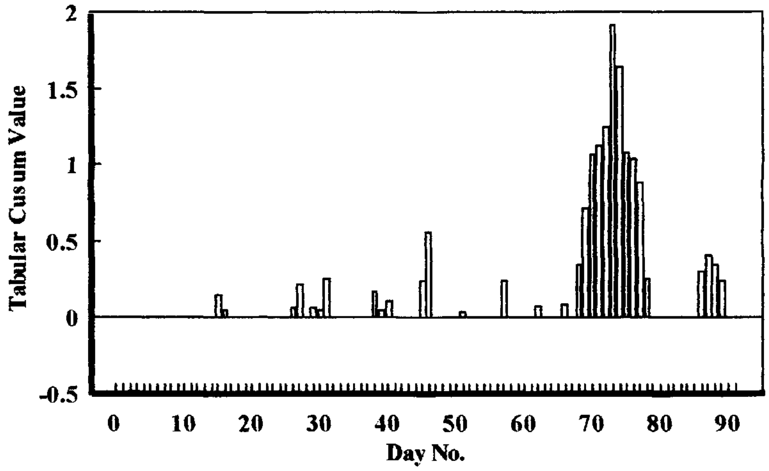
Plot of differences between the observed and reference value for ATP on dairy plant

B



Cusum chart of the cumulative sums of the differences in ATP levels plotted at (A) above

C



Single-sided Tabular Cusum plot based on log transformed data from (A) above

Figure 60–14 Control Charts for ATP Measurements on a Milk Filling Machine Mandrel. *Source:* Data from G.D. Hayes, A.J. Scallan, and J.H.F. Wong, Applying Statistical Process Control to Monitor and Evaluate the Hazard Analysis Critical Control Point Hygiene Data, *Food Control*, Vol. 8, pp. 173–176, 1997, Butterworth-Heinemann.

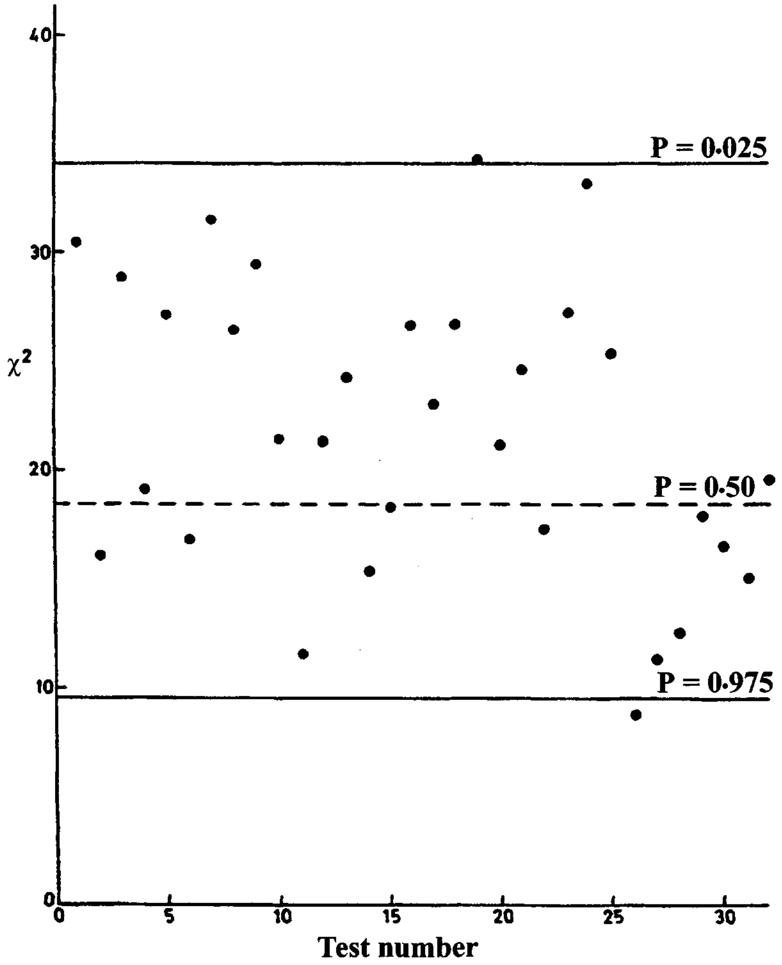


Figure 60–15 Shewart Control Chart for χ^2 Values Derived from Colony Counts of *Clostridium botulinum* and *C. sporogenes*, Based on 20 Replicates per Analysis. *Source:* Data from R. Spencer, Variability in Colony Counts of Food Poisoning *Clostridia*, *Leatherhead Food RA Research Reports No. 151*, © 1970, Leatherhead Food Research Association.

the variance of counts from 0.334 to 0.075. Increasing the size of individual analytical samples reduces the intersample variation and ensures that each individual sample unit is more representative of the production lot than are smaller samples, provided that the entire analytical sample is macerated to produce the initial sample dilution. Comminution of the sample unit prior to taking the analytical sample unit will also give a more even distribution of microorganisms in the analytical sample and will therefore reduce intersample variation. Similar findings have been obtained on replicate counts on a diverse range of foods and other biological materials. The effects are particularly noticeable when examining dry particulates (eg, cereals, nuts, dried fruits) or multicomponent foods.

The data in Table 60–19 also show that the mean colony counts appear to increase as a consequence of both increasing sample size and comminution. This apparent increase is an artifact arising from use of the mean log colony count rather than the log of the average colony count; the latter is a measure of the true count.[31] An estimate of the average count $(\bar{A})$ is given by:

$$\bar{A} = \frac{1}{n} \sum_{i=1}^{n} x_i$$

Table 60–18 Number of Sample Units Required To Obtain Reasonable Accuracy for Average Counts If the Log Counts Are Normally Distributed with Variance s^2, Assuming That the Means of the Log-Normally Distributed Random Variables Are Themselves Approximately Log-Normally Distributed To Obtain Conservative Estimates of the Number of Sample Units Required

Variance of Log-Normal Counts (s^2)	*95% Sure Of 90% True Average*	*90% Sure of 50% True Average*
0.01	14	1
0.04	60	1
0.09	155	3
0.16	338	6
0.25	699	12
0.36	1,451	25
0.49	3,137	54
0.64	7,266	123
0.81	>	309
1.00	>	854
1.21	>	2,607
1.44	>	8,834
1.69	>	>

Source: Reprinted with permission from D.C. Kilsby and M.E. Pugh, The Relevance of the Distribution of Micro-organisms within Batches of Food to the Control of Microbiological Hazards from Foods, *Journal of Applied Bacteriology*, Vol. 51, pp. 345–354, © 1981, Blackwell Science, Ltd.

where $x_1, x_2, x_3, x_4, \ldots\ldots x_i$ describes the level of organisms in each analytical subsample from sample 1 to sample *i*. Because the counts are log-normally distributed, the counts for each subsample will be distributed about the geometric average count or mean log count ($\bar{x}$),

$$\text{where} \qquad \bar{x} = \frac{1}{n} \sum_{i=1}^{n} (\log x_i)$$

The relationship between the mean log count and the log average has been defined[38] as:

$$\log \alpha = \mu + \ln 10\sigma^2 / 2$$

where σ^2 is the population variance of the log counts, log α is the log of the average count, μ is the population mean log count, and ln10 is the natural logarithm of 10 (2.3026). It is clear from this equation that the log average will always be greater than the mean log unless the variance is zero. Because, for any set of analytical data, s^2 is an estimate of σ^2 and $\bar{x}$ is an estimate of μ, then an estimate ($\bar{A}$) of the average count (α) can be derived by substituting s^2 and $\bar{x}$ in the equation above:

$$\log \bar{A} = \bar{x} + 2.3026 s^2 / 2 = \bar{x} + 1.1513 s^2$$

Because the log average count ($\bar{A}$) is the better measure of the true count, it can be concluded that when comparison is made with criteria, it is the log average count that should be used in order not to change the baseline as a result of examining samples of different size or from different stages of a production process—alternately, the sample size should be defined and kept constant and different criteria defined for each stage of a manufacturing process.

Table 60–19 Effect of Analytical Sample Unit Size and of Manufacturing Process on the Mean and Variance of Replicate Bacterial Colony Counts on Meat

Product or Process Stage	*Number of Sample Units (n)*	*Sample Unit Weight (g)*	*Mean Log Count ($\bar{x}$)*	*Population Variance (s^2)*	*Log Average (Log $\bar{A}$)*	*Calculated Log Average* (Log α)*
Frozen boneless beef	60	20	5.404	0.274	NA†	5.719
	20	100	5.504	0.185	NA	5.718
	20	500	5.664	0.026	NA	5.694
Thawed boneless beef	20	20	5.934	0.334	6.514	6.599
Minced beef	20	20	6.389	0.108	6.512	6.521
Bowl chopped beef	20	20	6.475	0.075	6.563	6.570

* for equation see text
† NA = data not available

Source: Data from D.C. Kilsby and M.E. Pugh, The Relevance of the Distribution of Micro-organisms within Batches of Food to the Control of Microbiological Hazards from Foods, *Journal of Applied Bacteriology*, Vol. 51, pp. 345–354, © 1981, Blackwell Science, Ltd.

60.5.2 Quantification of Data from Two-Class Sampling Plans

Calculation of Maximum Probable Level of Contamination. If salmonellae are present in a food in low numbers and are heterogeneously distributed, they are more likely to be detected if the variance is low, such as would be the case after bowl chopping of meat.[31] Hence, the effects of analytical sample unit size and pretreatment as described above will apply equally to the situation where salmonellae are sought using presence or absence tests. Habraken, Mossel, and van den Reek[21] and others have shown that salmonellae are not distributed randomly in dried milk-based products. Theoretical calculations, reinforced by examination of many tons of skim milk-powder products, showed that salmonellae occurred in nonrandomly distributed clusters. A similar situation also may occur, for instance, where postpasteurization contamination occurs. This creates enormous problems for drawing representative samples because the likelihood of detecting the presence of one or more clusters of salmonellae in a very large bulk of product is very low. The only realistic remedy is to examine a large number of individual analytical units and to ensure that the size of the analytical samples is sufficiently large. The sampling plan of Habraken et al.[21] recommends testing 300 × 2.5g sample units per lot. This is much more stringent than the most stringent of the ICMSF[24] sampling plans for dried milks (60 × 25g sample units per lot; Table 60–4) because the latter is based on the assumption that salmonellae are distributed randomly.

On the unlikely assumption that the incidence of defective units (eg, salmonellae) does occur randomly, it is possible to derive estimates of the probable maximum incidence or level of contamination for various probability levels (P). The equation, derived from the binomial distribution, assumes that organisms are distributed randomly, that n samples are examined, and that no positive results are obtained and is given by:

$$P = 1 - [1 - (d/100)]^n$$

where d = actual % incidence of defectives in a lot. This will be true provided that n is a small fraction of the total lot size. The equation may be rearranged to give the % defectives for a given probability (P) and number of sample units (n):

$$d = 100[1 - \sqrt[n]{(1-P)}]$$

For instance, if 30 samples were analyzed and all were found to be negative, then at a probability of P = 0.95, the calculated maximum incidence of contamination is given by

$$d = 100[1 - \sqrt[30]{(1-0.95)}] = 9.5\%$$

Assuming that each analytical sample weighs 25g, then the maximum likely level of contamination is given by (9.5/100) × (1000/25) = 3.8 salmonellae kg^{-1}. By contrast, if only 5 × 25g samples had been tested, again with negative results, the 95% probability of maximum contamination would have been 18 salmonellae kg^{-1}. Some examples of the effects of different sample unit size and number are given in Table 60–20.

Calculation of Most Probable Number from Presence or Absence Tests. If one or more positive results are obtained using a sample plan with $c = 0$, then the lot would be considered to have failed the test. However, a two-class sampling plan is essentially a series of replicate analyses that constitute an MPN test at a single inoculum level. It is possible, therefore, to generate an MPN from such data to give an indication of the probable incidence of contamination. The calculation uses the number of negative sample units at a single dilution level (ie, no viable organisms detected in the analytical sample) to derive the probability of occurrence of zero defects, so that:

$$P = z/n = e^{-vm}$$

where P = probability of occurrence of zero defects, v = the quantity of analytical sample unit tested in each replicate, z = the number of negative sample units, and n = the total number of sample units tested with a true density of m organisms per unit of sample tested. This can be rewritten as

$$m = -(1/v)\ln(z/n) = -(2.303/v)\log(z/n)$$

where ln and log are the logarithmic values to base e and base 10, respectively, and the other terms are given above.

Assuming that in a test of 30 × 25g replicate sample units, one positive result for salmonella is obtained, then the MPN of contamination can be derived as 1.36 salmonellae kg^{-1} with a 95% CI of <1 to 3.6 salmonellae kg^{-1}:

$$m = -(1000/25)\ln(29/30) = -40 \times -0.0339 = 1.356 \text{ salmonella kg}^{-1}$$

Similarly, two positive results would give an MPN of 2.76 salmonellae kg^{-1} (95% CI <1 to 6.1 salmonellae kg^{-1}). It should be noted that if no positive results had been obtained, then the MPN would be 0 salmonellae kg^{-1} with a maximum likely level of contami-

Table 60–20 Calculated Maximum % Defectives, Maximum Likely Levels of Contamination, and MPN of Organisms Based on a Two-Class Sampling Plan (Presence/Absence Tests) for Different Sample Numbers (*n*) and c = 0, Assuming Random Distribution of Microorganisms

		Maximum % Defectives at P =*		*Maximum Likely Level† of Organisms kg^{-1} at P =*		
Number of Samples (n)	*Size of Sample Unit (g)*	*0.95*	*0.99*	*0.95*	*0.99*	*MPN kg^{-1}†*
5	25	45.1	60.2	18.0	24.1	8.9
10	25	25.9	36.9	10.4	14.8	4.20
15	25	18.1	26.4	7.2	10.6	2.80
30	25	9.5	14.2	3.8	5.7	1.36
60	25	4.8	7.4	1.9	3.0	0.67
100	25	3.0	4.5	1.2	1.8	0.40
300	2.5 25	1.0	1.5	4.0 0.4	6.0 0.6	1.30 0.13
600	2.5 25	0.5	0.76	2.0 0.2	3.0 0.3	0.70 0.07

* Assumes all (*n*) tests are negative; values derived using equation in Section 60.5.2.

† Assumes one positive test out of *n* sample units; values derived using equation in Section 60.5.2.

nation, at 95% probability of 3.8 salmonellae kg^{-1}. Further examples for different sample numbers and sizes are shown in Table 60–20.

This procedure can be applied in many different circumstances. For instance, in an assessment of spoiled product, it can be useful to have an indication of the likely level of initial contamination. By counting the number of unspoiled sample units out of a large number of units examined (possibly the whole lot or a statistical sample of that lot), it is possible to derive an MPN for contamination of the lot using the proviso that each spoiled unit contained at least one viable organism. If this procedure is done on samples drawn from a range of lots manufactured on successive days or on different process lines, it is possible to assess the extent of contamination.

For instance, following the recall of a significant volume of bottled beverage due to reports of bursting bottles, it was possible to assess the extent of yeast contamination over a time continuum and then to relate this back to changes in manufacturing practices in the production plant concerned. It also permitted recalculation of the required process pasteurization conditions, based on the typical maximum level of yeast in the product prepasteurization, the known heat resistance of the organism, and the MPN of organisms in the packaged product. This eliminated pasteurization failure as a cause of the spoilage problem and focused attention on postpasteurization contamination. In this investigation, the typical level of contamination was found to be about one viable yeast cell per 10 liters of packaged product—a level that could not be detected with any reasonable confidence by microbiological sampling and testing of final product and reinforces the need for HACCP.

Implications of Sampling on the Laboratory Detection of Pathogens. Analysis for pathogens in food often requires a resuscitation, or pre-enrichment procedure, followed by elective or enrichment culture and finally detection of specific reactions on differential laboratory media (see Chapter 62). Individual colonies, presumptively identified as the organism sought, will then be selected for confirmation of identity using appropriate procedures. It is essential to recognize that such a sequential detection and isolation procedure involves sampling at each stage. For instance, unless sufficient time is allowed in pre-enrichment, the organism sought may not have recovered sufficiently to be detected and will not grow when transferred into the enrichment broth. Similarly, if only limited growth occurs in the enrichment culture, a loopful of culture removed to streak onto a differential medium may not contain the selected organism even if it is present at low levels in the enrichment culture. This would not be true if sufficient time had been allowed so that the enrichment culture contains a high proportion of the organism being sought. Picking of a limited number of colonies from a diagnostic medium for further examination also is influenced by statistical distribution unless a monoculture exists.[28]

60.5.3 Compositing of Samples

Laboratory protocols sometimes recommend that when many individual sample units are to be analyzed, some of the individual analytical units can be composited to reduce the total amount of testing to be carried out. Suppose that the ICMSF sampling plan[24] for salmonellae in skim milk powder is used with 60 × 25g sample units and that the sample units are composited to give the equivalent of 10 × 150g quantities. What will be the effect on the probability of detecting salmonellae? The probability of accepting a given incidence of defective units, if all test results are negative, is derived from the testing of 10 sample units each of 150g rather than 60 samples of 25g. From Table 60–6 it can be seen that if a lot has a true incidence of defectives of 10%, then $P_a = 0.35$ for $n = 10$, whereas for $n = 60$, $P_a = < 0.01$. Calculation of the maximum incidence of defectives at $P = 0.95$, using the equation in Section 60.5.2, shows that for $n = 10$, $d = 25.9\%$; for 150g samples, this is equivalent to a maximum of 1.7 salmonella kg^{-1}. For 60 samples each of 25g, $d = 4.9\%$, which is equivalent to 1.9 salmonellae kg^{-1}. Hence, there is a 35% chance of accepting a lot with greater than 1.7 salmonellae kg^{-1} if all tests are negative on 10 composite 150g samples, but there is less than 1% chance of accepting a lot with more than 1.9 salmonellae kg^{-1} if tests on 60 individual 25g samples are all negative. Clearly, testing of composite samples is much less discriminatory.

It is also important to recognize that if one or more of the composited sample units gives a positive test result, it is not possible to identify which of the 10 original sample units may have been contaminated. In practice, this may not be of importance if the result of a test is merely to accept or reject a lot; but if the incidence of positive sample units is required, then sample compositing will limit the availability of data. A compromise is to inoculate each sample unit individually into a pre-enrichment broth and then to composite appropriate quantities of the incubated pre-enrichment cultures at the time of inoculation of the enrichment medium. In this situation, any positive result can be checked by retesting the residual pre-enrichment cultures (provided they haven't been discarded).

60.5.4 Continuous Sampling

Devices for continuous removal of samples of solid or liquid foods are sometimes used to draw representative samples of material in process. One system for powdered milk products cited by Habraken et al.[21] enables a total of 300 × 2.5g samples to be drawn during the production of a lot. This was demonstrated to be a more efficient manner of monitoring for contamination than was testing a smaller number of larger samples drawn from the finished bulk product. However, such sampling devices are not normally suitable for testing lots of bulked or prepacked final product. Other forms of continuous sampling devices are used in the screening of consignments of peanuts and other bulk commodities for aflatoxin and other forms of contamination; they are normally applied during the mechanical transfer of product from a ship or other bulk container to a silo.

Sampling devices for liquid products take a continuous proportion of the liquid passing the sampling port, thereby yielding discrete (eg, timed) samples for analysis. Others pass a continuous stream of liquid through a captive membrane filter that can be removed manually at predetermined intervals for laboratory incubation and examination. However, such membrane samplers are frequently unsatisfactory in practice.

60.5.5 Environmental Sampling

The taking of environmental samples may be an important part of any regular production surveillance operation. Other than in relation to sampling plans for plant hygiene, for instance, based on swabbing clean, sanitized surfaces, most environmental sampling is predicated on random sampling of air and/or product residues from around the plant (eg, from air filters). Appropriate sampling plans can be defined on a plant-by-plant basis whereby a range of source materials identified in a HACCP study as being important are sampled on a regular basis for evidence of specific contamination.

60.6 CONCLUDING REMARKS

Sampling foods for compliance with criteria is done to enable decisions to be made as to the quality or acceptability of the product for use, sale, or consumption. Part of establishing the criteria is defining the required sampling plan; unfortunately, many published criteria[2–4] do not define sampling plans or are based on the use of only a single sample unit. The low level of repeatability and reproducibility of most microbiological methods means that little confidence can be placed in the results of such testing other than in relation to the specific sample unit analyzed.[30] Clearly, a single sample unit cannot be truly representative of a large product lot either in production conditions or in trade. Unfortunately, even the sample plans that are used frequently, such as those of ICMSF,[24] cannot provide a high degree of confidence in the data because the number of sample units specified in these sampling plans are significantly lower than the number regarded to be statistically necessary for rejection of defective material, and avoidance of rejection of sound materials, at a specified AQL level.[9]

Where the same product is made repeatedly under defined production conditions, the application of SPC systems adds considerable value to microbiological test data derived, for each production lot, from a limited number of sample units. However, such testing and analysis are retrospective and should be seen merely as an aid to safe food manufacture, which should be based on the principles of HACCP. Food products in trade cannot be examined effectively other than by end-product sampling, and for such products an effective sampling plan is essential, but even then no guarantee can ever be given that a product is safe or acceptable for consumption. Failure to detect organisms of public health significance may be due to inadequate sampling, imperfect methodology, and/or imperfect technique. Application of recommendations for safe food handling and control will always be more effective than excessive microbiological analysis of final products since quality and safety cannot be tested into a product.[1,13,23–27] Nonetheless, where testing is required, it must always be predicated on an understanding of those statistical factors that can affect both the analysis and the interpretation of the data.

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Appendix 60–A

Terms Used in Statistical Analysis and Sampling

The following definitions relating to statistical analysis and sampling for microbiological analysis are provided for ease of understanding of the text.

Acceptance sampling—Use of a predetermined sampling system to decide whether or not a "lot" meets defined criteria of acceptance. There are two types of acceptance sampling scheme: *Attributes sampling* and *Variables sampling.*

Acceptable Quality Limit (AQL)—A measure of the incidence of defective product that would normally be considered to be acceptable to a producer.

Analytical sample—see Sample

Attributes Sampling Plan—A sampling plan that considers the results of analysis purely in terms of defined attributes, which are graded as acceptable, marginally acceptable, or unacceptable according to the criteria of the plan.

Coefficient of variation (CV)—The ratio (as a %) of the standard deviation to mean value:

$$\mathrm{CV}(\%) = 100\frac{\sqrt{\text{variance}}}{\text{mean}} = 100\frac{\sqrt{s^2}}{\bar{x}}$$

Confidence interval (CI)—A measure of the spread of results about a mean value obtained (approximately) by multiplying the standard error of the mean by 2, for a 95% CI, or by 3, for a 99% CI. More accurate calculations of CIs are described in standard statistical texts.

Consignment—A quantity of product destined in commerce for delivery to a particular recipient, usually consisting of multiple units from one or more lots.

Consumer's risk—The probability that a lot will be accepted on the basis of analysis of sample units in accordance with a defined sampling plan, when in reality the lot as a whole is defective relative to the stated criteria of acceptability.

Defective—Not suitable for the purpose intended.

Frame—That portion of the lot from which the sample units are drawn; in practice, samples may sometimes be taken only from the accessible portion (or frame) of a lot.

Lot (or batch)—A quantity of product produced on a single occasion under a single set of production conditions and bearing a single identification number that relates to and defines the specific production run. The lot number might include data relating to the particular factory, production line, retort, date, or production time.

Lot Tolerance Percent Defective (LTPD)—Sometimes also called Rejectable Quality Limit (RQL), the LTPD is a measure of the incidence of defective product that is unacceptable to the producer.

Mean (arithmetic average) (usually designated $\bar{x}$, but sometimes designated m or λ)—The arithmetic mean, or average, value $\bar{x}$ obtained from a set of data values is calculated from the sum of the individual values (x) divided by the number of independent replicate tests (n):

$$\bar{x} = \left(\sum x_n\right)/n \text{ where } x_n = \text{individual values of } x \text{ from } n = 1 \text{ to } n$$

Mean log colony count (geometric average)($\bar{x}$)—Estimated by adding the logarithmic transformed counts (log x), dividing by the number of replicate counts (n), and then taking the antilog; or by taking the nth root of the product of the individual values (x) multiplied together, where n = the number of independent replicate tests.

$$\begin{aligned}\bar{x} &= \text{antilog}\left((\Sigma \log x_n)/n\right)\\ &= \text{antilog}\left((\log x_1 + \log x_2 + \log x_3 + \ldots\log x_n)/n\right)\\ &= \sqrt[n]{(x_1 \times x_2 \times x_3 \times \ldots x_n)}\end{aligned}$$

Log average colony count (log $\bar{A}$)—Estimated by taking the $\log_{10}$ of the sum of the individual counts divided by the number of replicate counts (n)

$$\log \bar{A} = \log((\textstyle\sum x_i)/n)$$

Operating characteristics (OC) curve—A statistically derived curve that relates the likelihood of accepting or rejecting a lot based on the expected incidence of defective units and the sampling plan used.

Population (microbiological)—The number of microorganisms, total or of defined species or group, dispersed within a defined quantity of food and detectable by defined methods.

Population (statistical)—The aggregate of units about which an inference is to be made from analytical results on a series of sample units; for practical purposes, the population may be taken to imply the total individual sample units within a lot.

Probability (P)—A measure of the likelihood that an event will occur; for instance, a probability of 95% (P = 0.95) indicates that an event would be expected to occur on average on 19 occasions out of 20, or conversely, that an event would be expected not to occur on average more than once in 20 occasions.

Probability of acceptance—The likelihood that a lot of known quality would be accepted on the basis of analysis in accordance with a defined sampling plan.

Producer's risk—The probability that a lot of acceptable quality will be rejected on the basis of analysis of sample units in accordance with a defined sampling plan.

Random sample of size n—A collection of n sample units obtained in such a way that every individual sample unit has an equal chance of selection, hence avoiding sampling bias.

Sample—The total number of individual sample units drawn at random from a lot that is to be tested in accordance with specific sampling plan and methods.

Sample (analytical)—The amount of material taken from a sample unit for analysis.

Sample unit—An individual portion or container of food taken as part of the sample and from which one or more analytical units are to be drawn for analysis.

Sampling plan—A statement of the criteria of acceptance to be applied to a lot based on examination of the required number of sample units by defined analytical methods.

Standard Deviation (s)—A measure of the spread of data relative to the mean. The term is obtained by taking the square root of the variance (s^2).

Stratification—A technique for dividing consignments into logical groupings such as can sizes, grades, boxes, or cases, that control known sources of variation. It may also be used to divide large lots or consignments into smaller groupings for purposes of sampling (see also *Frame*) and may be used deliberately when prior knowledge suggests that a lot or consignment is of uneven quality.

Variables Sampling Plan—A sampling plan in which the statistics (eg, mean and standard deviation) derived from analysis of sample units are used as criteria against which to assess the quality of the product (See also *Attributes Sampling Plan*).

Variance (s^2)—A measure of the spread of results in a set of n analyses; estimated by determining the sum of the squares of the difference between individual results (x_n) and the mean value ($\bar{x}$) and dividing by the number of degrees of freedom (n–1), ie:

$$s^2 = \frac{\Sigma(x_n - \bar{x})^2}{(n-1)}$$

61

Detection of Microorganisms in Foods: Principles of Physical Methods for Separation and Associated Chemical and Enzymological Methods of Detection

Anthony N. Sharpe

61.1 Preparation of samples for analysis
  61.1.1 Nonquantitative methods that yield images of microbial contamination
  61.1.2 Semiquantitative methods
  61.1.3 Methods for quantitative removal of microbes
  61.1.4 Microbial separations as part of rapid methods of analysis
61.2 Detecting microbial cells or their activity
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  61.3.8 Gas liquid chromatography
61.4 Concluding remarks
References

61.1. PREPARATION OF SAMPLES FOR ANALYSIS

Because microorganisms are so small, they present very small analytical signals even when present in foods at levels (eg, 10^6 g^{-1}) typically acceptable for spoilage flora. At common levels of regulatory interest (1 cell 25 g^{-1}), whole single pathogen cells such as salmonellas represent only 10^{-13} of the sample, and probably only 10^{-18} to 10^{-19} of it if one is searching for a nucleotide sequence characterizing salmonellas. Thus, detecting and quantifying microorganisms in foods present challenges of varying severity. One may:

- extract whole target cells from the food and identify or quantify them by culturing and using their phenotypy such as serological or enzymological properties
- detect them directly in the food by analyses conferring on the target cells some property (eg, light emission, radioactivity) that is detectable against the background of the food
- extract a cell component (most probably DNA or RNA) to provide a relatively consistent base for analysis, regardless of the food/species starting point.

However, reliable microbe detection depends on moving the microbes from the food into the detection system. Even today there are no sampling methods capable of removing microbes quantitatively from foods. For spoilage flora, the best that can be hoped for is reproducible removal by the sampling method. Pathogens at low levels may exist in pores, cracks, biofilms, or other areas inaccessible to suspending procedures, and it is virtually essential to pre-enrich the whole sample to give them time to multiply and escape into a menstruum before attempting detection.

The mechanism by which microbes in foods or other substrates are detached by the sampling technique is essentially unknown and certainly much more complex than might appear at first sight. A puzzling feature of microbial detachment is that during sampling (rinsing, stomaching, or blending)

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the concentration of suspended microbes quickly reaches a plateau; however (where circumstances permit), repeating the process with a change of diluent liberates more microbes, frequently with barely reduced concentration. If the sampling process is repeated many times, the summed counts may yield a value much higher than suggested by a single processing,[28,130,134,191,193,226,260] and my unpublished observations.

Some authors have assumed that microbial release follows a first-order reaction, as though the microbes in suspension are able to inhibit the detachment of others; however, it is difficult to believe that the Law of Mass Action would have a significant effect on this type of behavior—other effects are surely more important. Shaking, swabbing, stomaching, or blending all cause a degree of tissue destruction, and even during the sampling process, microbes may reattach to newly exposed surfaces, or to components of the sampling device (for example, the swab fibers or the stomacher bag). Whatever the mechanism, one should not assume that the yield of microbes from any sampling technique (even blending) represents more than a small fraction of their actual level. This consideration is vitally important for the development of rapid pathogen detection methods because techniques are compared against traditional pre-enrichment methods, for which a single viable cell may eventually grow out into a broth and be detected, regardless of its state in the sample. If a rapid detection method requires preparation of a sample suspension, suspending less than all of the pathogen cells will clearly result in poor performance by the new method when cells are present at a low level.

Current microbe-suspending technologies (rinses, stomachers, blenders) produce large volumes of suspension (100 to 250 ml) to analyze sufficiently large samples of food. Yet powerful separation and identification techniques (immunomagnetic beads, flow cytometers) useful for ultrarapid analyses cannot handle more than a few milliliters of suspension. Reducing the primary suspension to a volume of a few milliliters or less is difficult because (except in very small number of circumstances): (1) it is likely to have a very variable composition in terms of the types and properties of the target microbe, of the types of particles suspended in it, or the degree of association of the target microbes with those particles, and (2) because the large volume of the suspension prevents the use of expensive reagents. There is an urgent need for new methods to generate primary suspensions and to purify and concentrate microbes in suspensions, both more suited to rapid detection procedures. In various parts of the world, work is in progress on the use of lectins, immunomagnetic particles, standing ultrasonic waves, dielectrophoresis, and other techniques to separate pathogens from foods.

The development of microbial dispersal methods predates food microbiology, although older procedures such as shaking samples with sand or even pouring molten agar over samples have largely disappeared. Methods producing an image of microbial contamination (eg, "agar contact" or RODAC*-type plates, sticky tapes) are useful but should not be used for estimating microbial numbers. For enumerative work, methods yielding reproducible and representative suspensions are essential. A short account of nonquantitative, semiquantitative, and quantitative methods of microbial detection follows.

61.1.1 Nonquantitative Methods That Yield Images of Microbial Contamination

The simple, inexpensive testing methods described in this section have a unique utility in monitoring hygiene or troubleshooting microbiological problems, but must not be regarded as providing quantitative information on microbial loads. Much evaluation work carried out in earlier years in hopes of using such methods as substitutes for excision and blending methods or swabbing showed very poor correlations. Colony counts on RODAC plates and dip slides are often less than 1% of the counts obtainable by a rigorous technique of microbial removal, dilution, and plating. Often it is only possible to infer that one surface is more contaminated than another, or that over time the level or type of contamination on a surface changes; however, virtually no other microbiological techniques have the ability to demonstrate, for example, a source of contamination such as a hairline crack in a piece of equipment—this is the real power of imaging techniques.

Agar contact plates, commonly known as RODAC or as Rosier-Pantaleon plates in France, are small (usually 65 mm diameter) sterile plastic dishes with arched lids, filled to the "convex meniscus" level with agar medium. They are simply opened, pressed carefully against the test surface, reclosed, and incubated. RODAC plates were introduced in 1964 for bacteriological monitoring in hospitals,[92] found widespread use in the food industry, and have been amply described and tested.[70,242,247] They are available prefilled with a variety of agar media through several suppliers or may be obtained empty for filling with special media. Their ease of use and the "contact prints" they show of contamination are well suited to the teaching of hygiene practices. Because they may be used onsite by relatively unskilled staff with little need for other equipment and returned to the laboratory for incubation, they are the ultimate in simplicity and convenience. The data must, however, be treated with the same caution as all methods in this section.

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*RODAC, replicate organism direct agar contact.

Dip slides, also called AIPC (agar immersion, plating, and contact slides) by Mossel[161,162] are flexible plastic slides, covered on both sides with sterile agar media and enclosed in sterile, plastic tubes. They are obtainable custom-filled with specific culture media, frequently differing on the opposite sides. In addition to dipping into fluids, they can be pressed against surfaces in the manner of RODAC plates and are useful for microbiological testing of water, milk, foods, and manufacturing environments.[84,90,98,99,147,163]

Agar syringes and sausages are simple and inexpensive versions of agar-contact techniques, popular years ago because they permitted extensive monitoring of surfaces in food-processing facilities, but they have been replaced in plant environments by adenosine triphosphate (ATP) and other determinations. The agar sausage technique[246] used an agar-filled sausage skin, the exposed end of which was pressed against a test surface and then sliced off with a sterile knife and incubated in a petri dish. The agar syringe[197,267] is similar, except that agar was held in a large diameter syringe from which the end had been removed. Reduced moisture loss gave agar syringes a better shelf life than agar sausages. These are very underrated techniques, capable of providing much monitoring information around a plant at absolutely minimal cost.

Sticky tapes also can be used to remove microbial cells for direct microscopy or for plating and incubating in the manner of an agar contact technique. The techniques are not quantitative, but interesting specific effects occur; for example, viable amylolytic *Arthrobacter* spp. adhere to starch-based films[105] while dead bacteria or other species fail to do so.

61.1.2 Semiquantitative Methods

Swabs are essentially nondestructive to carcasses and equipment and yield minimal debris with the removed microbes, but their efficiency of microbe removal tends to be very unreproducible and usually well below the level needed in a quantitative sampling technique. Until recently, no swab techniques, except perhaps for the highly standardized Danish swab method[173] or the total object swab[160] yielded even reproducible fractions of the surface counts provided by more rigorous methods. In one of the most detailed and controlled studies on swabs,[106] a wet and dry swab method for carcasses and carcass meats gave summed counts from the two swabs ranging from 1% to 24% (average 10%) for fresh beef carcass, 27% to 52% (average 37%) for fresh mutton, 13% to 67% (average 44%) for fresh pork, and 25% to 89% (average 39%) for chilled pork belly, compared with counts from the excised, blended surface.

61.1.3 Methods for Quantitative Removal of Microbes

Improved Swabs. The limited release of foodborne microbes into suspension during sampling, and the possibility that newly exposed surfaces or the sampling device itself traps microbes liberated from foods, suggests that reproducibility problems of swabs stem from the high concentration of microbes built up at contact surfaces and inability to distribute them uniformly through even the small diluent volume held by the swab. The Rotorinser (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada) is an "improved swab." It holds a large volume of diluent (10 ml for a 50 cm^2 area) in a disposable, open-cell, urethane-foam cylinder that scrubs the test surface while compression and rotation of the cylinder pumps liquid repeatedly through the foam to bring microbes from the test surface into equilibrium with the diluent. The foam cylinder retains diluent when the process is stopped so that the instrument can be used on surfaces at any angle. In trials on pork skin and beef carcass surfaces, the Rotorinser removed a greater proportion of microbes than did excision followed by stomachering. For example, based on summed counts from double sampling (rotorinsing followed by stomachering, or stomachering followed by stomachering), the instrument gave removal efficiencies of 0.93 and 0.98 for 30- and 60-second operations, respectively, for pork, compared with 0.86 for a Stomacher.[225] On beef carcass tissue, the Rotorinser gave removal efficiencies of 0.74 to 0.91 for 30- and 60-second operations; however, stomachering was too destructive to the samples to permit a value to be obtained for comparison. The Rotorinser appears to provide a more representative analytical sample than swabbing or even excision and stomachering.

Spray Methods. Sprays also are relatively nondestructive and yield low debris levels. A spray gun that used air at a pressure of 35 to 207 kPa (5 to 30 psi) to spray diluent over the surface of samples, with washings being collected as they ran off, yielded bacteria counts as high as, or higher than, a blender.[34] Other workers have claimed various successes with similar techniques.[11,97,201] A "flush gun"[128,129] was capable of removing 50% of the surface bacteria count on pork or beef in 1 minute; with higher surface counts (above 5×10^7 cm^{-2}), a 90% efficiency was claimed. A portable instrument,[253] developed into the PASS (portable automatic spray system), is available from PBI International, Milan, Italy. It is limited to vertical or near-vertical surfaces.

Ultrasound and Vortex Stirring. Some early studies[103,149,189,195,196] indicated good removal of microbes from hard surfaces by insonation. For some food samples in glass tubes, agitated either in a vortex stirrer or suspended in an ultrasonic tank, removal of microbes compared well with conventional blending and yielded bacterial suspensions with a very low debris content.[218] For intact beef, peas, and green beans, total counts obtained by insonation were about 50% of those obtained by bladed blender. Insonation was less effective for prawns, which protected surfaces from the energy source (1.5 $\log_{10}$-cycles lower) and very poor with a compressed, comminuted meat (4 $\log_{10}$-cycles). Vortex stirring in glass test tubes was very effective, counts of total aerobes, *Staphylococcus aureus*, and *Escherichia coli* not differing significantly and being within ±25% of bladed blender counts for beef, sausage, chicken, prawns, cod, and green beans, and were 50% lower for peas and 80% lower for bacon.[218] Sonication conditions must be a compromise between effectiveness and lethality from cavitation. The small sample size and need to sterilize a container are problems for both techniques, and both seemed to have lapsed into obscurity.

Blenders and Stomachers. I am unable to find original articles on bladed or turreted blenders in food microbiology, but their use appears in many standard or official methods and they were long assumed to give "quantitative" suspensions of microbes from foods. Problems of noise, the need to clean and sterilize after use, overheating, and high levels of debris in suspensions have been problems. This led to development of the Stomacher, which processes samples in disposable plastic bags, massaging and crushing them under two paddles. It causes less tissue disruption than bladed blenders, and smaller temperature rises in the suspension.[219]

Through its convenience, the Stomacher replaced bladed blenders in many laboratories, but suspicion about its less destructive action led to many performance evaluations. The original evaluation[219] used peptone water diluent, but following further study,[221] addition of 1% Tween 80 surfactant usually is recommended to improve microbial yields from foods of moderate to high fat concentration. An exception is for materials such as butter when stomachering for membrane filtration. In the presence of surfactant, these foods emulsify and become unfilterable; in the absence of surfactant, they stick to the walls of the stomacher bag, leaving a clean suspension.[223,224]

In eight published studies, the Stomacher yielded about the same count as a bladed blender; in six there was no difference and in about 20 the count was slightly lower. The Stomacher gave higher counts than a blender for:

- poultry carcasses[1]
- cheese—and with no observable effect of citrate[7]
- aerobes and Gram-negative bacteria from frozen ground beef[111]
- in impedance measurements with meats[27]
- trichinella[138]
- hard and soft cheeses[166]
- meats, seafood, and cooked foods, although cheese, powdered egg, and milk gave lower counts[259]
- lung smears of *Pneumocystis carinii.*[252]

There were no significant differences between Stomachers and blenders for:

- casein[169]
- ground beef, celery, or wieners[211]
- counts of bacteria on chicken skin[248]
- *Trichinella*[250,251]
- high-fat dairy foods[254]
- (essentially identical) counts of *Listeria monocytogenes* from Colby cheese.[278]

The Stomacher gave slightly lower counts of

- coliforms and *Salmonella* in fatty foods[2]
- *Salmonella* from frog legs,[3] in a general comparison[2]
- in meat products and salads,[10,12,14] from raisins, hard cheese, and wheat grains and for molds in meats and sausages[21] for counts from human colon walls[43]

- *Bacillus cereus, Streptococcus fecalis, Yersinia enterocolitica*, and *Staphylococcus aureus*[50]
- *Listeria monocytogenes* in beef tissues, although Tween 80 increased the number of bacteria recovered from fat tissue,[51] in meats, with or without Tween 80[62]
- trichinae[79]
- *Clostridium perfringens*,[93] from chicken carcasses[121]
- aflatoxin in egg powder[138]
- from sputum[159]
- Enterobacteriaceae in chicken,[164] and in seafoods, pork, hams, and sausages.[168]

General reviews of Stomacher performance can be found in literature,[85–87,89,94,133,138,139,202,216,232] while others cover safety aspects,[39] improved bags with filters,[134] and control of foaming.[238]

A recent advancement over the paddle-type blender is called the Pulsifier (Microgen Bioproducts Ltd., Camberley, Surrey, UK). It accepts samples in disposable plastic bags; however, instead of kneading and crushing samples, it applies a combination of shock waves and intense agitation. Because microbes usually exist on surfaces or within easily accessible structures, "pulsification" efficiently disperses them. In a comparative trial[81] based on total aerobic counts from 96 samples of representative foods, the average ratio Pulsifier:Stomacher was 1.4:1. At very low count levels, total counts by Pulsifier were approximately twice those obtained by Stomacher, were significantly higher for samples with $<10^5$ cfu g^{-1}, not significantly different for samples with 10^5 to 10^7cfu g^{-1}, and slightly lower for levels above this. Except with precomminuted foods Pulsifier:Stomacher pulsified suspensions contain less food debris; for example, for celery and carrot total count ratios were 1.3:1 and 2.5:1, respectively, but pulsified suspensions were clear, while stomachered ones contained enough debris to interfere with pipetting.[81] Counts of coliforms (85 food samples) and *Escherichia coli* biotype 1 (36 food samples) by Pulsifier did not differ significantly from those by Stomacher.[222] All of the pulsified food samples filtered better through hydrophobic grid membrane filters (Section 61.1.4) than did stomachered ones, although the improvement varied with the food. For example, the ratio of filtration rates for Pulsifier:Stomacher varied from 12:1 and 10:1 for suspensions of beef liver and broccoli, respectively, to 1.4:1 and 1.3:1 for chili powder and feta cheese, respectively.[222] The improved filtration rates indicate a reduced level of suspended debris in suspensions prepared by pulsifier compared with paddle-type processors. This may prove beneficial for techniques in which interference by food components is a problem (eg, polymerase chain reactions [PCRs], or ATP bioluminescence); even its performance with total counts might reflect reduced interference and better visibility of colonies. Because the Pulsifier does not crush samples, hard objects (rice, bones, gravel) cause less bag-damage. A removable, transparent door allows the action to be viewed and improves accessibility for cleaning.

61.1.4 Microbial Separations As Part of Rapid Methods of Analysis

In the current race to develop faster screening methods, much attention is being paid to procedures for separating and concentrating microbial cells from the interfering components of food suspensions by means of physical or chemical methods, rather than waiting for multiplication to increase the microbial signal. Some of these techniques are well established; others are still in an experimental stage. The most pressing problem is that of initial treatment of the primary suspension. For rapid separation, it is essential for the separating or capturing agent to contact the target microbe quickly. For large volumes, the capturing agent must be inexpensive enough to be used in large amounts so that it can essentially fill the sample volume; this places severe limitations on the range of materials usable. Once the sample has been brought down to 1 ml or less, more sophisticated reagents can be considered. A theoretical treatment of microbial separations suggests that it is much more efficient (ie, faster) to separate the target microbe by using multiple steps of lesser efficiency, than a single step.[217]

Membrane Filtration Separations. This is a prime separation method, capable (not necessarily without difficulty) of separating microbial cells from food tissues, and of concentrating microbial cells for detection. Membrane filtration can lower limits of detection in direct microscopic methods by several orders of magnitude; for example, in the direct epifluorescent filter technique (DEFT). The DEFT-type procedure has been improved even further[257,258] for direct detection of *Escherichia coli* O157 in juice and meat at levels down to 10 cells g^{-1}. The DEFT, and a new instrument based on ATP bioluminescence (Microstar), are covered in more detail later in this chapter.

The success of membrane filter methods depends strongly on the filterability of food suspensions. Unprocessed foods (raw meats, fish, vegetables) generally yield suspensions that filter easily. With increasing levels of processing, addition of gums and fillers, food suspensions become less filterable, and dairy products often present problems. The problem usually is not difficult to overcome. Much work on the filtration of food suspensions was carried out during development of the hydrophobic grid membrane filter (ISO-GRID HGMF, QA LifeSciences Inc., San Diego, California), which is used for microbial enumeration and for detection of pathogens. Through its ability to "streamline" conventional incubation procedures on growth media, and its very wide (10^4:1) linear counting range, the HGMF provided a basis for many rapid counting and detection methods. At least seven of these have become official action procedures of the Association of Official Analytical Chemists International (AOAC), including counts of total aerobes, coliforms, fecal coliforms, *Escherichia coli*, a combined coliform/*E. coli* count, and yeasts and molds, together with detection procedures for *Salmonella* spp. and *Escherichia coli* O157:H7.[63–66,135,180,181,223]

To yield a linear count over many orders of magnitude, the HGMF exploits the most probable number (MPN) principle. Its grid of 1,600 (40 × 40) tiny grid-cells is equivalent to a tube-MPN assay of 1,600 tubes all at one dilution; the user does not attempt to count individual colonies, but rather, scores each grid-cell as positive or negative according to whether it contains any evidence of growth of the target organism. After obtaining the score (X), the MPN of colony forming units (growth units) that inoculated the HGMF is given by:

$$\mathbf{MPN = N \log_e ((N-X)/X)}$$

where N is the number of grid-cells (1,600 for the ISO-GRID HGMF). The MPN value can be much greater than N, since the "colony overlap" that prevents conventional agar plates from yielding accurate counts at much above 200 colonies, is accounted for by the logarithmic equation.[220] Automated counters, or microprocessor-assisted manual counters eliminate or simplify the counting step.

Although the HGMF relies on a relatively conventional incubation and growth of colonies, the MPN principle it exploits is now being used in a number of rapid or simplified counting procedures. For example, the Simplate (IDEXX Laboratories, Inc, Brantford, Connecticut) is based on passive distribution of a sample suspension into MPN wells, and the MicroStar system (Millipore Corporation, Bedford, Massachusetts) uses a hydrophobically partitioned membrane filter to apply the MPN system in an ATP bioluminescence-based rapid counting procedure (see Section 61.3.3).

For microscopy, where the ability of cells to multiply is not important, digestion with trypsin and Triton X-100 surfactant efficiently removes unwanted debris.[42,184,186,204,276] Where it is necessary to preserve the viability of cells, Triton X-100 should be avoided, in favor of Tween 80. Table 61–1 indicates the type of treatment to employ if a suspension is difficult to filter. The book by Sharpe and Peterkin[224] provides a more complete source of information on membrane filtration of foods.

Immunomagnetic Separations of Bacterial Cells and Toxins. Microbes can be caused to become ferromagnetic or paramagnetic by processes such as directly adsorbing submicron particles of magnetic iron oxides on their surfaces or treating them with Erbium (Er^{3+}) ions,[279] or precipitating ferromagnetic ions on their surfaces.[60] However, the most popular methods involve immobilizing microbes, in suspension, on tiny (2.8 to 4.5 μm) paramagnetic polystyrene beads (Dynabeads, Dynal, A/S, Oslo, Norway) or primed, silanized magnetic iron oxide particles (BioMag, Metachem Diagnostics Ltd., Piddington, Northants, UK) by means of lectins or, particularly, monoclonal antibodies. By using antibody-coated particles, other target species, such as microbial toxins, may also be made magnetic. The great attraction of immunomagnetic methods lies in the speed and simplicity with which the target species may then be separated by means of a powerful magnet (permanent- or electro-). Techniques may be as simple as collecting a pellet of magnetic cells, washing them by resuspending and recollecting, or more sophisticated processes such as thin-layer magnetophoresis.[177,207] The separated cells may be detected by simply plating them on normal growth media, by electrical impedance methods, or by PCR, where immunomagnetic separation can remove inhibitory materials,[75,175,270] enzyme-linked immunosorbent assay,[125] or other methods. Immunomagnetic methods can capture and detect dead microbes or those that are severely damaged and undetectable by standard culture techniques.[145]

In general, separation works best with fairly high levels of immunomagnetic particles. For salmonel-

Table 61–1 Enzyme Treatments To Increase the Filterability of Foods

Food	*Enzyme*
Skim milk	None
Raw milk	None
Butter and margarine	None
Other milks	Trypsin
Spray-dried milks	Trypsin
Cheeses	Trypsin
Spray-dried cheese powders	Cellulase or Protease
Sour cream	Diastase
Yogurt	Trypsin
Ice cream	
without stabilizers	Trypsin
containing gums	Hemicellulase
containing cellulose	Cellulase
Egg—liquid or powder	Trypsin
Raw or cooked meat or poultry	Trypsin
Shrimp	None
Oysters	Trypsin
Citrus juices	Pectinase
Fruit puree	Pectinase
Raw vegetables	None
Flour	None
Rice	None
Chocolate	Amylase
Breakfast cereals	Cellulase
Cake mixes	Amylase
Lecithin	Lecithinase
Food colorings	None
Gums	Hemicellulase
Infant formula	Trypsin
Sodium caseinate	Protease
Nut meats	None or Protease

Note: Suspensions of foods in this table frequently filter sufficiently well through membrane filters without treatment to permit common types of microbial count. In cases of difficulty, or where larger volumes are to be filtered to improve limits of detection, suspensions (1:10) are treated with 1% or 2% enzyme for 20 minutes at 35° C.

lae and *E. coli* O157:H7, particle concentrations as high as 10^6 to 10^7 ml^{-1} have been used.[76,230,264] Incubation periods of 10 to 60 minutes are required, and attachment increases with time. However, with unduly long incubation, nonspecific attachment and heavy contamination with nontarget microbes reduce the effectiveness of immunomagnetic beads as a selective analytical step. Surfactants such as Tween-20 or protamine reduce nonspecific attachment. Some limitation of immunomagnetic beads is found in the small volume analyzable because of the short range of a magnetic field, and a tendency for less-than-quantitative attachment even with great excesses of bead to target cell. Plate counts from immobilized microbes tend to be lower than the actual number of cells owing to a tendency for multiple attachment of cells; a common rule is that one colony may represent up to six target organisms.[230]

Interest in immunomagnetic beads has grown rapidly. A range of techniques and kits is now based on separating and concentrating suspended microbes, either directly from the initial suspension or after a short enrichment period to provide earlier detection.[30,31,44,113,140,141,230,231,256] Commercial magnetic particles are available primed with lectins or secondary antibodies to facilitate methodology. Among the methods developed are: detection of *E. coli* O157:H7 at a level of 1 cell ml^{-1} in meat after overnight enrichment;[172] microbiological confirmation of enterohemorrhagic *Escherichia coli* during an outbreak;[30,275] detection of salmonellae at levels of 10^2 cells g^{-1} in various foods.[231] Detection using anti-*Salmonella* Dynabeads was as good as three traditional procedures with food samples,[145] although an immunomagnetic bead method for detection of *Salmonella* in naturally contaminated feeds detected only 33% of positive samples, compared with up to 100% for four other methods.[72] K-serovars of *Vibrio parahemolyticus* were separated from foods, and *Vibrio hemolyticus* was identified in foods incriminated in food poisoning.[256] At least one commercial kit (VICAM Listertest, VICAM, Watertown, Massachusetts) is available for detecting or enumerating *Listeria monocytogenes.* For further information on use of immunomagnetic beads, see Chapter 63.

Centrifugation. Despite being a cumbersome step, centrifugation will probably continue to have a major role in microbial separation. For example, centrifugation at 2,000 g for 10 seconds prior to estimating biomass by ATP measurement removed virtually all meat particles without decrease in bacterial count.[235] Density gradient centrifugation can remove food debris without loss of bacterial count; a 15 minute method using colloidal silica clarified food suspensions,[8] and the automated density gradient apparatus of the Bactoscan instrument (see below) also allowed concentration of food-related microbes.[136] Sedimentation Field-Flow Fractionation separates pure bacterial cultures and is probably applicable to foods; cells are injected into an open, unpacked channel, first sedimented by a low (5 to 10 relative centrifugal field [RCF]) centrifugal field, then fractionated by the parabolic fluid-velocity field as diluent passes through the chamber.[215]

Standing Wave Ultrasound. Reflection of sound waves in a tube to yield standing waves causes suspended particles to concentrate at nodes, which can then be moved by varying the frequency.[37,91,158,269] It is tempting to think of detaching cells from a test surface, then moving them off to a place of collection by the same ultrasonic forces. Particle size, concentration, energy levels, and other factors affect efficiency and there has not yet been a practical application of the technique.

Dielectrophoresis. When an electric field is applied between plate and pin electrodes, slightly conducting particles suspended in liquid in the nonuniform field migrate to or from the pin electrode. The direction of movement depends on the relative conductivities of particle and liquid. Unlike electrophoresis, which requires direct current (DC), the dielectrophoresis effect occurs regardless of whether the electric field is alternating current (AC) or DC. Electrode assemblies are barely larger than the cells they are used with, and may be fabricated on silicon semiconductor chips. Complex electrode arrays can also rotate cells, and particles modify the applied fields, allowing electronic analysis of the situation.[82,102,182] While the small size of dielectrophoresis units is unlikely to allow their use for processing primary suspensions of foods, the ability to combine them with electronic signal processing and control suggests that they have a useful potential for separating microbes in food microbiological analysis.

Biphasic Partitioning. Bacteria, viruses, and other bodies partition themselves between the phases of aqueous biphasic systems (eg, of polysaccharide and gelatin mixtures), permitting some degree of separation.[17,142,150] Not only can *Salmonella* and *Escherichia coli* be separated, but so can rough/smooth mutants of *Salmonella typhimurium*.[236,237] Commercial applications in food microbiological analysis have not yet been described although biphasic separation is under evaluation by a number of groups.[178]

61.2 DETECTING MICROBIAL CELLS OR THEIR ACTIVITY

61.2.1 Microscopy

Older direct microscopic techniques suffered problems of poor limits of detection (10^7 cells g^{-1} or higher), distinguishing live cells from dead ones or from tissue organelles, and of relating microscopic counts to plate counts. A prime drawback has been that prolonged use of microscopes rapidly leads to fatigue and loss of accuracy. However, modern microscopic techniques can provide very rapid information on levels (even species) of microbes in foods, costs per test are low, automated instruments are appearing, and mounted preparations take up little space and may be stored for reference. Moreover, image analyzers improve continually in their performance and affordability and, through their ability to tirelessly examine large numbers of stained preparations, will eliminate some of the present drawbacks. At times, the information provided by microscopy may shed light on the history of a product in a manner that plate counts are incapable of. For example, although the plate count may become zero after a pasteurization step, the microscopic count may remain high because the enzymic activity that would normally destroy dead microbial cells also has ceased; a high count of dead microbial cells, however, can indicate that a food material was of general poor quality.

Even many years ago, procedures were developed to reduce drawbacks of direct microscopy; for example, examining microcolonies formed during short incubation periods,[80] or techniques in which microcolonies were detected by optical scanner[24] or by Coulter Counter.[25] The DEFT was a major advance, permitting concentration of organisms on membrane filters to improve limits of detection, and using a nuclear stain to distinguish live from dead organisms. Use of fluorescent antibodies to allow detection of specific foodborne microbes began with the Fluorescent Antibody (FA) Technique for salmonellae.[69] Although very useful in permitting screening for salmonellae in a much shorter time than previously possible, the method suffered from inadequacies of the antisera of the day (cross-reactivity, low sensitivity) and required a very precise technique and careful training to produce reliable results. For these reasons, it fell from popularity. An automated FA Technique actually was developed, probably several years prematurely;[165] however, labelled monoclonal antibodies have lent great power to DEFT-type techniques.

Direct Microscopic Counts. In these venerable yet still useful techniques, a known quantity of food is spread thinly over a defined area of a microscope slide or counting chamber, dried, fixed, defatted, and stained if necessary, and examined. Individual cells or clumps of cells are enumerated in sufficient microscopic fields to obtain a precise count. The commonest procedure, for the examination of milk, is ascribed to Breed.[26] The Howard mold count[101] requires a special counting chamber; it was developed for estimating rot in tomatoes but, like the Breed method, may be used under suitable conditions and with suitable precautions for other foods. Both procedures require considerable practice to achieve accurate results.

Direct Epifluorescent Filter Technique (DEFT). The DEFT enumerates viable and/or nonviable microbes. With a sensitivity limit of 6×10^3 bacteria ml^{-1} in milk and in other foods, it is essentially a technique for estimating spoilage flora, though there are instances where it may be possible to indicate potential hazards. Its great value is in yielding data in 25 to 30 minutes. It was developed for use with raw milk but now is used with many other foods; for example, for detection of molds in vegetable products[187,272] and evaluation of starter activity for fermented foods where rapid production of acid or other preservative agents is essential to prevent outgrowth of pathogens. The DEFT needs a minimum of equipment besides the epifluorescence microscope, and costs per sample are low. In its manual form it is labor intensive and involves prolonged microscopic work, but automated versions are now available.

The improved sensitivity results from concentration of cells on 0.2 μm membrane filters. Ability to distinguish live and dead bacteria results from staining with the nucleophilic fluorochrome acridine orange, which fluoresces at different colors in DNA and RNA during different phases of growth. Generally, viable cells fluoresce orange-red, while dead ones fluoresce green, although this is not an absolute rule. Food samples are digested with trypsin and Triton X-100 to improve their filterability, filtered through a "track-etched" membrane filter, stained with acridine orange, and sufficient fields must be examined under the epifluorescence microscope to give a reliable count. The DEFT requires only a moderate level of skill, but images must be interpreted with caution; for example, bacterial cells from heat-treated (blanched) products tend to retain their RNA, appearing to be "viable." The book by Pettipher[183] is the only one of its kind available, but a review[184] gives more up-to-date details.

A completely automated DEFT system (Cobra 2024, Biocom, Paris, France) is available with a sample throughput rate of 150 samples h^{-1}, and claimed repeatability better than the manual DEFT between 2×10^4 and 1×10^6 cfu ml^{-1}, with a lower counting limit better than 2×10^4 cfu ml^{-1}. The Cobra was basically intended for milk grading but gave good correlations with plate counts and manual DEFT counts for meats and fish.[188] Related automation includes an automated direct fluorescence microscope system (Autotrak) for continuous analysis of water and effluents,[212] which examines 140 specimens h^{-1}, providing results in 30 seconds, with good correlation coefficients for microbial counts in the range 10^4 to 10^8 cfu ml^{-1}.

The Bactoscan instruments automatically carry out an enumeration procedure rather similar to the DEFT. The method is an official procedure for obtaining data for "payment by quality" purposes in several European Union (EU) countries where the Class 1 limit for raw milk is 10^5 cfu ml^{-1}. In the Bactoscan III (Foss Electric, Hillerod, Denmark), milk samples are first digested to lyse somatic cells, then concentrated and separated by gradient centrifugation, rather than membrane filtration. After being stained with acridine orange, the sample is deposited on a rotating disk that is scanned by the computerized fluorescence microscope. A count takes approximately 7 minutes. Bactoscan counts tend to be higher than plate counts, partly because psychrotrophs are included.[45,153,199,243,244]

Related Direct Microscopic Techniques. The Viablue 2 procedure is similar to the DEFT procedure but uses the aniline blue stain Viablue 2 counterstained with rhodamine or Rose Bengal.[155] According to the authors, it gives a clear-cut differentiation between live and dead cells of bacteria and yeasts (dead cells fluoresce orange, viable cells green), allowing rapid determination of viable cell counts of bacterial isolates from milk, and *Listeria monocytogenes* isolated from soft cheese. Unlike acridine orange-based procedures, there was no reversal of staining with heat-killed cells.

Another membrane filter-based microscopic count (DINTS), described as suitable only for milks and derived from the DEFT, uses vital staining by means of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), which produces crystals of black/purple formazan inside actively metabolizing cells.[18] Unlike DEFT, it requires only a standard microscope, plus the filtration accessories. DINTS requires only about 15 minutes, and the requirement that cells be actively metabolizing makes it more specific than DEFT.

Pathogen Detection by Direct Microscopy. *Escherichia coli* O157:H7 has been directly enumerated in milk and apple juice within 1 hour by an antibody-DEFT method.[257] After digestion with trypsin/Triton X-100 in the usual manner, the milk was stained with fluorescein-labelled anti-O157 polyclonal antibody; apple juice filtered adequately without digestion. Limits of detection in both cases were about 10^3 ml^{-1}. The antibody-DEFT technique also directly detected *Escherichia coli* O157 inoculated into beef at levels down to 16 cfu g^{-1};[258] a trypsin/Triton X-100 digest was passed through a 5 μm membrane prefilter prior to the 0.2 μm collection membrane. Detection of *E. coli* O157:H7 at this level would require two days or more by conventional methods, or slightly over one day by an immunostaining procedure on HGMFs.[245] While obviously capable of producing a more rapid result, detection at the lowest level by the antibody-DEFT method required the examination of 200 microscope fields.

Flow Cytometry. The technique of flow cytometry[157] owes its origins to classical microscopy, with the sample being viewed and assessed automatically. The basic principle is that the sample is forced to flow in a narrow, well-defined, liquid stream through a focused laser beam. As each organism passes through the beam, it is imaged onto photosensors, which measure optical changes. These changes are related to the size, shape, and fluorescence properties of the cell. Through the use of appropriate fluorescent labels, it is possible to measure total nucleic acid content, viability, surface antigens, or rRNA, for example. The power of the technique is derived from an ability to measure each cell one by one as it passes through the interrogating laser, and to measure a number of different parameters for each cell simultaneously by using multiple detectors. Owing to the cost and complexity of the instrumentation required, flow cytometry has not achieved the widespread application to food microbiology that it has to biomedicine; however, with the availability of cheaper bench-top instrumentation, such as the ChemFlow (Chemunex S.A., Maisons-Alfort, France) and the Bryte HS (BioRad, Hemel Hempstead, England), this situation is gradually changing.

The sample must be prepared in a liquid suspension, and prefiltering at around 50 μm is necessary to prevent large particulate matter causing flow problems. Typically, the sample flows through the cytometer at about 1 μl $second^{-1}$ and up to 5,000 cells $second^{-1}$ may be detected. However, the ultimate effectiveness of the system is limited not by optical and electronic considerations, but by sample preparation and the specificity of the fluorescent reagents used. Simple protocols with, for example, the nucleic acid stain ethidium bromide give a count of total number of organisms present in the sample. Although highly accurate for laboratory cultures, this method is less convincing when applied to meat or dairy samples. Flow cytometry has been successfully applied to the detection of spoilage organisms (yeasts) in soft drinks, where there exists a high background of other biological matter.[185] Through the use of fluorescently labelled monoclonal antibodies, it is possible to detect one or more target cell types within a mixed population. Clarke and Pinder[35] assayed two different salmonellae, even in the presence of a large excess of *Escherichia coli* cells, in samples of milk and eggs, down to a limit of about 10^3 cells ml^{-1}, with an assay time of 40 minutes. Fluorescently labelled oligonucleotides represent another form of highly specific probe suitable for flow cytometric analysis. They have been applied to microbial consortia in sewage, for example,[146] although their undoubted potential with food matrices has yet to be realized.

A number of protocols have been developed for the flow cytometric assessment of cell viability.[112] The Chemunex ChemChrome system is designed for use in industrial environments: a nonfluorescent precursor is cleaved by the action of intracellular enzymes in viable cells to release a fluorochrome, which is concentrated within the cells, provided their outer membranes are intact.[127] By combining ChemChrome with their monoclonal antibodies, and using three-color flow cytometric detection, Clarke and Pinder[36] improved their previously quoted detection limit by an order of magnitude. Also using ChemChrome, it is possible to detect so-called viable but nonculturable organisms; this technique was applied by Firth[74] to study *Vibrio vulnificus* in shellfish.

In its most elaborate form, flow cytometry combined with electrostatic cell sorting has been used to monitor *Cryptosporidium* oocysts in water.[265] Because there is potential for misidentification, the putative oocysts are automatically sorted onto a slide for confirmation of their identity by manual microscopy.

The use of flow cytometry combined with immunomagnetic separation of cells was shown to be a sensitive and rapid method for the detection of *E. coli* O157:H7.[214]

61.3 CHEMICAL AND BIOCHEMICAL TESTS

61.3.1 Bioluminescence

All animal, plant, and microbial cells contain the nucleotide phosphate ATP in relatively constant concentration. ATP can be measured within seconds at extremely low levels by a bioluminescent reaction in which oxidation of the substrate luciferin is catalysed by the enzyme luciferase. Originally, luciferin and luciferase were obtained from extracts of firefly lanterns;[154] both substances are now made from bioengineered organisms. The general procedure is to inject cellular extracts containing ATP into cuvettes containing luciferin and luciferase, with added magnesium ion at pH 7.4. Typically, the intensity of the luminescence rises to a maximum in milliseconds, then decays over a period of minutes. If luciferin and luciferase are present in excess, the maximum intensity of light emitted is proportional to ATP concentration. At least 90 ATP-based instruments of varying capability and price have been reviewed.[233,234]

Data for ATP content strictly relates to biomass, but can be related to numbers of target cells if the system has been previously calibrated for that organism. Microbes may be estimated through their ATP content; however, food analyses are complicated by the overwhelming excess of animal or plant tissue ATP, which decreases only slowly and incompletely when cells die.[216] The success of ATP determination for estimating bacterial numbers depends strongly on the efficiency of procedures used to separate bacteria from the food or destroy its intrinsic ATP before the assay. Although ATP data do not always correlate well with plate count, biomass is actually a better indicator of the stability of a product than is a count of colony forming units; for example, the total cellular mass of a *Streptococcus* chain better represents its metabolic potential than the single colony it produces on an agar plate.

Because they are present in greater numbers, ATP-based bioluminescence has found use for detection of spoilage organisms and monitoring starter cultures.[13,216,262,274] Until recently it had not had significant application to the detection of pathogens. Its greatest use is currently for measuring biofilms and food residues on surfaces,[15,126,213] and for this purpose portable instruments with disposable swab/reagent packs, which make them very easy to use, are now available for cleaning efficiency testing.

In addition to ATP-based bioluminescence tests, procedures based on nonluminescent microbes that were bioengineered to luminesce have been described. By recombinant DNA techniques the *lux* gene from a naturally luminescent bacterium, *Vibrio fischerii*, was inserted in phages or bacteria; the energy source is reduced flavin mononucleotide ($FMNH_2$) rather than ATP. Experiments such as insertion of the lux gene into P22 phage, which was then applied to *Salmonella typhimurium* culture, causing the phage-infected bacteria to become luminescent within 30 to 50 minutes have been reported.[239-241] The sensitivity and specificity of bacteria/phage reactions suggested a potential for shorter than normal enrichment times. Experiments in which luminescing bacteria became dark when injured, stressed, or killed quickly demonstrated the presence of antimicrobial residues or the activity of biocide preparations; for example, luminescent lactic bacteria quickly indicated the presence of antibiotics and bacteriophages in whole milk, a test relevant to monitoring starter activity;[240] however, no commercial applications of these techniques appear to have materialized.

61.3.2 Estimating Microbial Numbers by ATP Determination

Since even 10^6 bacteria g^{-1} represent less than one part per million of a food, the tissue ATP represents a massive excess requiring that special separation measures be used if the test is to be applicable to practical levels of bacteria. Separation methods tend to be based on differential filtrations, incubation with ATPases (apyrases), or combinations of these. The first step in differential filtration is to filter out food tissue cells because they are usually larger than microbial cells. Microbial cells are collected by filtration, washed to remove free ATP, and then their cell walls are perforated chemically to liberate the microbial ATP. In apyrase treatments, the enzyme acts on free ATP and is unable to attack ATP contained in the microbial cell; after destroying as much free ATP as possible, the microbial ATP is liberated and measured. While the theoretical limit of detection may be 1 to 10 cells in pure culture, it is difficult to reliably detect fewer than 10^5 g^{-1} of bacterial cells from most foods.

A typical method for estimating microbial numbers in fresh beef and chicken used a primary filter that removed 99.99% of nonmicrobial ATP, and a second that collected bacteria. The second filter was then treated with apyrase to remove further nonmicrobial ATP. Bacterial ATP was then extracted and measured. Quoted limits of detection were 5×10^4 cfu g^{-1} of meat sample, with bacterial levels predicted within ± 0.5 $\log_{10}$ of the actual aerobic plate count for more than 90% of samples tested.[137] Another procedure used for monitoring chicken quality in Hazard Analysis and Critical Control Points (HACCP) operations subjected chicken rinses to a coarse, then bacterial, filtration, followed by treatment with lipase. A limit of detection of 1×10^4 g^{-1} was reported, with a correlation coefficient of 0.85 against the standard plate count, and, at levels above 1×10^5 g^{-1} the ATP method accurately assigned 91% of samples to the correct quartile of a grading protocol.[15] Two methods for determining microbial loads from fecal contamination on carcasses are interesting. When carcass swabs were incubated with apyrase, total assay time was about 1 hour, but by using a filtration method assay time was reduced to about 5 minutes.[228] Correlation coefficients of 0.91 (beef) and 0.93 (pork) between microbial ATP and plate counts in processing plants, with limits of detection corresponding to $\log_{10}2.0$ (beef) and $\log_{10}3.2$ (pork) were reported.[228,229]

61.3.3 ATP Bioluminescence and Most Probable Number-Type Enumerations

Very recently, an instrument system was unveiled that potentially overcomes the tendency for poor correlation between ATP determinations and plate counts. The MicroStar Rapid Microbiology Detection System (Millipore Corporation, Bedford, Massachusetts) collects microbial cells on a hydrophobically partitioned membrane filter (HPMF) containing more than 600 compartments. The reader is referred to the HGMF (Section 61.1.4) for an explanation of how the MPN principle permits accurate counts over wider ranges than might be expected when one is familiar with counting colonies on plates or cells under a microscope. After filtration, the HPMF is sprayed with bioluminescence reagents and placed under an image intensifier, charge-coupled device (CCD) television camera, and image analyzer. The system "reads" the HPMF in approximately 2 minutes, and by analyzing the image can compute the number of microbial cells. The manufacturer claims that the system can detect large microbial cells, such as yeasts, directly; bacterial cells, being smaller sources of ATP, require incubation on growth media for a few hours in order to form microcolonies before being introduced to the bioluminescence reagents.

61.3.4 ATP Bioluminescence To Measure Surface Contamination and Cleaning

No matter how reliably cleaning and disinfecting solutions are made up, an important step to monitor during plant cleaning and sanitation is the thoroughness with which equipment surfaces have been cleaned. Dirt left on surfaces may harbor microbes that can recontaminate the product again as soon as the line is restarted. In particular, microbes in biofilms resist disinfectant solutions that kill unattached cells; thus, it is important to ensure that as much contamination as possible is removed from surfaces.

The great sensitivity of the luciferin-luciferase ATP bioluminescence reaction makes it an excellent way to detect unremoved material. The very problem that limits its use for enumerating microbes in foods (susceptibility to environmental ATP), becomes an advantage in monitoring surfaces in a HACCP operation. That is, although the ATP reading may not differentiate between microbes and food, the existence of a positive reading proves that the cleanup was not done properly (ie, the test is used to detect total ATP of both food and microbes). The test can be applied to a swab or other sample in 5 minutes; thus, a positive reading can be used to redirect the cleaning crew back to the area before processing is restarted.[16,126,213]

A number of instruments and kits specifically for hygiene monitoring are available. The instruments tend to be inexpensive, completely portable, have disposable sampling units containing swab, and all of the "chemistry" needed to buffer the test against interfering effects of sanitizers and other residues, then measure ATP after swabbing the test surface. Some instruments can be programmed to direct the user to critical control points or other points of interest, storing up to 5,000 test data that can then be downloaded to the laboratory computer.

61.3.5 Catalase Activity

While catalase activity measurements have been used for many years in monitoring the effectiveness of vegetable blanching processes,[5] and tests for catalase activity are commonly used during microbial identifications, several simple and rapid catalase tests are very useful in screening processes for incipient microbiological problems. At times it may be possible to calibrate the procedure in terms of microbial numbers; however, this is usually unnecessary.

For example, catalase activity in milk is cause for concern, regardless of whether it is due to microorganisms or leukocytes. A catalase test based on the time required for oxygen bubbles to refloat a sinking paper disk[32,273] is useful. The disk flotation method detects, in 2 to 3 minutes, for a few cents and with little skill requirement, gross microbiological problems in bulk milk or even the individual quarters of an udder. Reflotation time usually is checked visually, although an inexpensive instrument (Catalasemeter, Fisher Scientific, Pittsburgh, Pennsylvania) automates the timing if so desired. The test is useful for troubleshooting processing operations if spoilage microorganisms are catalase-positive; for example, catalase-positive contamination of vacuum-packed cooked turkey was detected at levels down to about 10^4 ml^{-1}.[52] At the lowest level, flotation took about 30 minutes. A simple test for field use described by the US Department of Agriculture for detection of flat-sour spoilage of canned corned beef simply required mixing 5 to 10 g of the product with 10 ml of 3% hydrogen peroxide solution and observing if bubbles formed.[48]

High levels of catalase activity are found in most tissues, and perhaps the most valuable use of catalase measurement resides not in tests of microbial presence but in monitoring critical control points of processes. Since catalase is destroyed rapidly above 65° C, any detectable catalase activity indicates lack of time/temperature control on, for example, a meat cooker. Chicken samples cooked to $\leq 69°$ C showed $\geq 99\%$ probability of positive catalase reaction, whereas samples cooked to endpoint temperature of 71° C had only a 3% probability of positive catalase reaction, and samples cooked to 72° C had no reaction.[4] A very simple field test based on formation of an oxygen foam when an undercooked meat sample is shaken with hydrogen peroxide and baby shampoo, requiring little skill and taking about 30 seconds, has been used for detection of underprocessing of roast beef.[67]

61.3.6 Limulus Amoebocyte Lysate Tests

Gram-negative bacteria, such as those primarily responsible for beef spoilage, are quickly detected in very small numbers by this test, which, though many years old, is still the simplest and most sensitive method available for detecting these organisms or, more strictly, the endotoxin (lipopolysaccharide) they produce. The reagent is derived from amoebocytes of the horseshoe crab *Limulus polyphemus*;[131] in the presence of purified bacterial endotoxins, it forms a gel similar to that from a positive coagulase test. As little as 0.03 ng ml^{-1} of purified endotoxin can be detected in 1 hour,[95] or as few as 50 Gram-negative cells.[38] Other workers have described limits of 0.05 ng endotoxin in 0.1 ml[132] and 0.5 ng in 0.1 ml,[271] and a variety of procedures.[107,114,115,205,277] These data are for relatively pure cultures, however, and in foods the limit is closer to 10^4 cells.

The basic gelation procedure requires minimal apparatus, and can be adapted to suit many foods; its cost compares favorably with that of making plate counts, but can be reduced still further if the test is miniaturized, for example, by using microtiter plates. In addition, since it detects both viable and nonviable organisms, and since the endotoxin survives common sterilizing processes, it affords some insight into the history of a product. Typically, 0.1 ml of suspected endotoxin solution or bacterial suspension in saline is mixed with 0.1 ml of *Limulus* amoebocyte lysate (LAL) in a test tube, allowed to stand 1 hour at 37° C, and inspected for gel formation. Various developments of the technique use turbidity or color reactions, and automated versions have been described.[114]

The test has been used successfully with ground beef,[107] sugar solutions,[95] and is probably useful for any products where the content of Gram-negative organisms is important. Detection limits range from 3.5×10^5 to 7.7×10^6 cells for various species isolated from meat, and within a closely defined situation, a reasonable correlation between LAL titer and numbers of Gram-negative organisms will probably be obtained. For example, LAL titers increased when ground beef was stored for up to 12 days at 5° C, and correlated well with counts of Gram-negative organisms.[107-111] False-positive results due to Gram-positive organisms or their products from a food should not be ruled out, and indiscriminate use of correlation factors to estimate bacterial loads is not recommended.

61.3.7 Determination of Microbial Metabolism

Electrical Impedance Measurement. An aqueous solution of neutral molecules conducts electricity no better than pure water. An ionic solution conducts electricity because ions move toward the electrodes in an applied electric field. A solution containing only large molecules such as carbohydrates and proteins has a low conductance, even if the solutes ionize, because macromolecules move slowly. Conductance (1/resistance) is a bulk property of the solution.

Adsorbed and immobilized molecules interacting with water molecules create electrical double layers at the electrode surfaces. Under an AC field, these electrical layers act as capacitors in series with the resistive bulk solution. The combined conductive and capacitative resistance is called the impedance. Through its capacitative component, impedance is frequency dependent. At low frequencies, an AC bridge measures mainly the capacitance, and at high frequencies, mainly the conductance; AC bridges operating at intermediate frequencies can measure both components simultaneously.

Microbial metabolism breaks down large molecules to small, mobile ions (H^+, acid anions), causing a progressive increase in the electrical conductance of the medium and changing electrode capacitance. Within limits, conductance change can be related to microbial concentration and the initial level of contamination. Within limits, capacitance change also reflects microbial number, though the effects are more complex. Selective detections, for example, of coliforms, yeasts, or *Salmonella* spp. can be made by varying the growth media and operating temperature.[55,57,73,88,167,209] Impedance or conductance measurement is now a popular means of estimating microbial contamination in foods or detecting pathogens. Several instrument systems are available, some measuring only conductance; others conductance and/or capacitance. Samples sizes range from 2 to 100 ml in reusable or disposable impedance cells, and the instruments handle at least 256 samples, are completely computer controlled, automatically interpret and record results, determine conformance with specifications, and can be connected into laboratory automation systems. Viable count estimations by impedance can often be made in 4 to 6 hours, there is no need to make dilutions, and optical clarity of suspensions is not important. While there are innumerable publications on applications of impedance, the book by Eden and Eden[57] is still the only comprehensive text on the subject.

Impedance changes due to bacteria only become detectable when cell concentrations reach 10^7 to 10^8 ml^{-1}; thus incubation is required for detection. The impedance detection time (IDT) reflects both the cells' initial number and their metabolic and multiplicative energies; thus, correlations with plate counts depend on the reproducibility of the microbial flora within a sample type. The technique is best used for "screening," or ascertaining whether or not samples fall within established specifications. From the regression line of IDT versus plate count for a specific sample type, and the coefficients of variation, a "gray zone" may be drawn around the maximum allowable plate count. Outside this zone, the impedance technique reliably indicates whether samples conform with the specification. Within the gray zone, one cannot be certain, and samples falling within it are re-analyzed by plate count. Because samples usually fall either well within or well outside a standard, only a minority of samples require re-analysis by plate count and overall analysis times and labor savings can be considerable.

Sterility testing of ultra-high temperature (UHT) dairy products, fruit juices and drinks, and aseptically packaged low-acid foods usually requires less than 48 hours.[41,208] The analysis usually involves pre-incubation of the sample (with or without added medium) at a temperature favorable for growth prior to transferring an aliquot to the impedance instrument for further incubation. Since impedance data reflect not only the count but the combined metabolic and multiplicative energy of the microorganisms, they can be better than plate counts at predicting shelf life. Impedance data predicted the shelf life of pasteurized milk better than did the Mosely test;[19,20,117] the technique requires a pre-incubation step at elevated temperature during which potential spoilage organisms multiply, followed by impedance monitoring in growth medium. A related use has been rapid detection of temperature abuse in broiler chickens.[206]

The conductance method for *Salmonella* based on the method of Easter and Gibson[56] was the first official method of the UK Ministry of Agriculture, Fisheries and Food,[192] and later achieved official action status of the Association of Official Analytical Chemists International.[5] It uses a nonselective preenrichment, followed by selective enrichment in media containing dimethylsulfoxide and mannitol during which the electrical detection is made. An alternative procedure[6] uses a bacteriophage specific for salmonellae. Other methods for detecting *Salmonella* have been developed based on media modifications,[170] or on impedance splitting (conductance and capacitance).[190] Direct impedance methods have been developed for other pathogens or indicator organisms; for example, for detection of *Staphylococcus aureus* in milk powders[192] or for fecal streptococci in milk powders.[167] Related uses include demonstration of antibiotic residue inhibition of lactic starters,[171,266] and mastitis in dairy herds.[119]

An interesting development is indirect conductimetry, where the electrodes are not immersed in the microbial growth medium but in a small vial of potassium hydroxide solution situated above it. Carbon dioxide evolved during metabolism reacts with the potassium hydroxide, reducing its conductance. The technique offers a more reliable means to detect yeasts whose products frequently do not increase conductance. It also permits high concentrations of salts to be used where selective media require them; detection of *Staphylococcus aureus, Listeria monocytogenes, Enterococcus faecalis, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Aeromonas hydrophila,* and *Salmonella* spp. have been described.[22,54]

Dye Reduction, pH Indicators, Fluorogens, and Chromogens

Dye Reduction and pH Indicators. Like impedance measurement, any signal reflecting the combined multiplicative and metabolic activity of organisms can be expected to be better than a plate count as an indicator of the microbial potential for causing undesirable changes in the product. Metabolic data may permit better monitoring of raw material quality or be a better predictor of shelf life, allowing better control over the distributed product. Metabolic measurements may be made on any microbial property relevant to the problem (eg, dye reduction, impedance, production of acid, H_2S, color, toxin) if the instrumentation exists. At the same time, substances indicative of microbial metabolism are useful diagnostic agents in plate and MPN types of enumerations.

Many colorimetric indicators ("dyes") are available to detect reducing systems generated by microbial metabolism in a growth medium. Evaluation methods based on reduction of resazurin and other oxidation-reduction indicators by microbes were used for many years by the dairy industry, until wider use of refrigeration reduced levels of mesophilic bacteria, the principal microbial reducers of these dyes. Despite the fact that there may be large populations of mesophiles, dye reduction has been less extensively used with other types of foods, mainly because of problems with the variability of strains and species of microbes in their ability to change the E_h of their substrate, the existence of strong reducing systems in some foods, and difficulties of obtaining uniformly colored backgrounds against which the color of the indicator can be read. Nevertheless, the speed with which it is possible to demonstrate microbial contamination can be attractive enough to override these potential drawbacks, and interest in these techniques is again increasing.

Exploratory work on a range of foods was carried out as early as 1939,[194] and methods can be as simple as, for example, pressing dye-impregnated filter papers against the surface of meat patties;[61] however, instruments with automated detection and data handling are now available. These tend to be less expensive than instruments for impedance measurement and can also be used with pH indicator systems or any others indicative of microbial metabolism. The Omnispec system (Wescor Inc, Logan, Utah), based on reflectance colorimetry of litmus, resazurin, bromocresol purple, or triphenyltetrazolium chloride, has AOAC official action status for estimation of viable counts. Advantages claimed over impedance methods include lower instrument cost and reduced sensitivity to temperature fluctuations or electrical interference. The indications are that reflectance colorimetry has the same range of applications as impedance microbiology, such as estimation of total plate count, selective groups of organisms (eg, coliforms, yeasts and molds, *E. coli*), shelf life, and antibiotic sensitivity.[203]

Resazurin, which changes from blue to pink (resorufin) to colorless (hydroresorufin) during reduction is the most "sensitive" Redox indicator in common use, in that it is reduced most rapidly by microbial action; however, it must be used with caution because it may be reduced by other tissue systems. Methylene blue reduction (blue to colorless) provides better correlations with plate counts than does resazurin, and a range of tetrazolium salts (colorless to red or purple) has found varied uses. Among pH indicators, methyl red (4.8–6.0), bromocresol purple (5.2–6.8), bromothymol blue (6.0–7.6), and neutral red (6.8–8.0) have found greatest use for lactic or deaminating microbes.

A typical procedure based on resazurin reduction establishes the acceptability of a raw meat consignment at the loading dock better than organoleptic tests, if regulations permit trucks to be detained for 2 to 3 hours. Through the determination of regression lines fitting dye reduction times to plate counts, microbial loads of meats can be estimated with reasonable accuracy.[100] Meat sample suspensions are prepared by rinsing or by Stomacher, which causes minimal disruption of the meat tissue and release of reducing systems ("reductones") from meat; the test is not suitable for comminuted meats. Unsterilized skim milk powder is added to the suspension to provide a white background and to minimize coloration by meat; a Lovibond or similar comparator is satisfactory for measuring color changes. A similar procedure was used to demonstrate contamination in cooked or cured meats and aid in solving processing-associated problems, a time to end-point of 3 hours corresponded to a level of 10^6 bacteria g^{-1} in the meat, within about ±0.45 log-cycles.[53] A slightly different procedure, in which swabs of beef steaks were rinsed out and incubated with resazurin (no milk powder) yielded a correlation coefficient of 0.94 against the aerobic plate count, and permitted meat to be graded as containing $\geq 10^6$ cfu cm^{-2} in 2.2 to 2.3 hours.[263]

Methods based on the reduction of tetrazolium salts to colored formazans have been used for many years. Various tetrazolium salts have been used in, for example, detecting microbial contamination in fish,[227] but because many formazans are red, they are less easily distinguished from meat juices, and have been less useful with meats. The vital stain INT that produces black/purple formazan inside actively metabolizing cells has been used for rapid microscopic counts in milk.[18] An ingenious technique for visualizing the pattern of microbial contamination on cured or cooked meats helped locate the source of a processing problem. Fresh production samples of sliced ham were pre-incubated at 30° C to 37° C for various times, then sprayed with INT and incubated in sterile pouches at 37° C for 15 minutes. A pattern of curved parallel red/purple formazan lines developed on the ham slices and a minute nick (the seat of contamination) was discovered on the revolving blade of the slicing machine as it passed through the ham.[25]

Fluorogens and Chromogens. Many substances yielding colored or fluorescent products after reaction with microbial enzymes or other components of microbes are used in various aspects of analytical microbiology. For example, fluorogenic dyes such as 8-anilino-1-napthalene-sulphonic acid and acridine orange are used to detect DNA as in DEFT (above), and pH-fluorescent indicators such as acridine or 7-hydroxycoumarin are useful for detecting microbial activities. Many chromogenic substrates are phenol derivatives; for example, *o*- or *p*-nitrophenols or 5-bromo-4-chloro-3-indolyl compounds, and many fluorogenic substances, such as 4-methylumbelliferone, are derived from coumarin.[143] The hydrolysis of fluorescein diacetate by microbes has been shown to be useful for estimating the aerobic plate count of meat. Swabs of beef steaks were centrifuged, and the resuspended pellets incubated 3 hours at 25° C with the reagent before measuring absorbence of fluorescein at 490 mm. A correlation coefficient of 0.92 against the aerobic plate count was found, and meats with levels of 10^6 cfu cm^{-2} were graded satisfactorily.[263]

Derivatives of β-D-glucuronide or galactoside have found extensive uses in kits and other methods as detectors of glucuronidase or galactosidase activities in rapid tests for presence of coliforms or *Escherichia coli*. The fluorescence precursor 4-methylumbelliferyl-β-D-glucuronide (MUG), which is metabolized by glucuronidase-positive microbes to a fluorescent umbelliferone, has been particularly popular. For enumeration on plates or membrane filters, MUG is less convenient; the need to count colonies under ultraviolet (UV) light and a tendency for diffuse staining are problems. Glucuronidase substrates providing visible color changes and insoluble colored products are preferable. Compounds such as 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (BCIG, also called X-gluc) and indoxyl-β-D-glucuronide (IBDG), both of which yield blue or purple deposits, have been found very useful.[77,78,200] For example, on BCIG-containing "ColiChrome 2" plates (RCR Scientific, Inc, Goshen, Indiana), coliforms grow as red colonies; *E. coli* as blue or purple.

The glucuronidase-based methods provide identification and enumeration systems comparing favorably with conventional *E. coli* methods, and reduce assay times to 24 hours or less. It should be noted that while 95% to 97% of *E. coli* strains are glucuronidase-positive, about 50% of *Shigella* and 30% of *Salmonella* strains show glucuronidase activity and also grow at 44.5° C, a common incubation temperature for *E. coli*.[48,71,120,179] Thus, without confirmation, tests may better be described as assays of hygienic quality rather than of *E. coli*. Interference by intrinsic glucuronidase activity has been reported from oysters[123] and some freshwater and marine plants.[49] In contrast, many pathogenic strains of *E. coli* (eg, *E. coli* O157:H7) are glucuronidase-negative.[249]

Galactoside substrates are typified by o-nitrophenyl-ß-D-galactopyranoside (ONPG), which is lysed by galactosidase-positive coliforms to yield yellow o-nitrophenol during incubation at 35° C for 24 hours. ONPG is frequently combined with MUG in kits to permit detecting both total coliforms and *E. coli* in one test; tubes turning yellow (coliform-positive) during incubation are further examined for fluoresence under UV light. Kits such as Colilert (IDEXX Laboratories Inc, Portland, Maine) and Coliquik (Hach Co., Loveland, Colorado) were developed for detecting total coliforms and *E. coli* in waters, for which application they perform satisfactorily.[33,174] They may be usable with foods, provided the sample does not contain interfering enzyme activity; in the Colilert system, ingenious "MPN plate" (Simplate) accessories permit instantaneous distribution of a sample-substrate mixture into 84 or 198 wells for MPN enumeration. Other galactosidase substrates include 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside or X-gal[143,144] and 4-methylumbelliferone-β-D-galactoside or MUGA.[29] In Rambach agar,[198] improved diagnostic capability is achieved by capitalizing on the ability of salmonellae to produce acid from propylene glycol. The medium contains propylene glycol, X-Gal, and neutral red; *Salmonella* colonies are bright red (neutral red precipitated by acid) while coliforms are blue (galactosidase activity) or violet (galactosidase activity plus acid formation). See Chapter 62 for further information on the use of chromogenic substrates for microbial detection.

Turbidity. The growth of microorganisms in a clear broth medium increases its turbidity and absorbance, a change that can be measured by spectrophotometer or nephelometer when cell densities exceed 10^6 ml^{-1}. Under well-defined conditions, the time required for the broth to achieve a certain absorbance is a reasonably consistent function of the inoculum, allowing estimation of the initial concentration of microbes. Homogenized foods are not ideal subjects for turbidimetry because their intrinsic turbidity signal may change with time (settling, enzymic activity); however, one can use turbidimetry for food suspensions if the inocula used are dilute enough to avoid excessive background noise.

A commercial turbidimeter (Bioscreen Analysis System, Labsystem, Helsinki, Finland) measures bacterial growth in a vertical optical pathway. Turbidity is read every 10 minutes at 620 nm. The system also contains a dispenser dilution unit and an incubator/shaker. Samples are incubated in microtiter plate-type vessels. The instrument stores the turbidity data and, after incubation, the individual growth curves of each well can be further analyzed. The system has been used to predict the number of cfu (total count) in a variety of food products including raw milk sausage, hamburger, and french fries and minced meat. Small sample volume may be a restrictive factor, and because of the need to dilute the sample 1:10 or 1:100, detection times were longer than by impedimetry; however, both capital costs and costs of disposable items were lower than for their impedance counterparts.[116,150–152]

To derive useful mathematical models of microbial growth (predictive models), a forbidding number of combinations of growth conditions may need to be studied before good models can be formulated. Turbidimetry has found considerable application in the derivation of data for predictive models on account of its simplicity, ease of set up, and the low cost per test. However, caution must be applied in calibrating systems in terms of cfu/ml because the turbidimetric signal depends on the phase of growth of cells, and may, in any case, only yield a ($\log_{10}$) linear response over a very limited range of cell concentrations.[96,156]

Turbidimetry or absorbance is also used in commercial identification systems; for example, VIDAS (Vitek Immunodiagnostic System, bioMérieux, France). For this instrument, identification cards and strips are available for most species of food-interest, such as Gram-negative non-Enterobacteriaceae, Enterobacteriaceae, staphylococci and micrococci, corynebacteria, *Salmonella, Yersinia enterocolitica,* and *Shigella* spp. Identification cards are inoculated from purified culture suspensions, placed in the instrument's incubator and absorbance is read automatically at regular intervals. For salmonellae, results are usually available in 4 to 6 hours, with good confirmation rates.[122] The method has AOAC official action status for foodborne *Salmonella* and for screening of non-*Salmonella* isolates. The instrument can be obtained as part of a complete system which also includes an impedance instrument (MicroTeam).

61.3.8 Gas Liquid Chromatography

It is outside the scope of this chapter to discuss the power and utility of gas liquid chromatography

(GLC) in identifying microbial species after their isolation and purification. Microbial identification by GLC depends on the fact that, if organisms are grown under strictly controlled conditions, cellular fatty acid composition in the 9 to 20 carbon atom range is a stable and highly conserved genetic trait[58] resulting in GLC profiles from which cultures such as *Vibrio vulnificus* and *Listeria* spp. may be identified.[268] Because the same fatty acids tend to occur in foods, direct detection or identification of microbes in foods is rarely possible; however, the sensitivity of GLC places it among the most interesting of chemical methods for estimation of microbial presence in foods.

Because the quantities of specific chemicals generated by individual microbial cells (particularly bacteria) are very small, relatively large populations of cells are required if they are to be detected by direct analytical methods. For this reason, despite its sensitivity, the use of GLC with foods is still confined to the estimation of spoilage flora. For example, the presence of D-lactate, acetate, and ethanol were correlated with deterioration of vacuum-packed or modified atmosphere-stored meats,[23,46,47,176] and the presence of D-alanine in fruit juices was a valuable indicator of microbial growth.[83]

Direct determination of volatiles in headspaces by GLC is particularly useful because no step is required to separate the analyte from food material. The technique was applied successfully to determination of total mesophilic bacteria or total coliforms in hamburgers. The method involved sequential analysis of carbon dioxide (CO_2) concentrations in equilibrium with headspaces of vials containing the inocula in suitable media. Linear relationships and excellent correlations between the time taken to reach a 5% level of CO_2 and the plate count ($r = 0.985$ and 0.966, for total count and coliforms, respectively) existed over a range of 10^2 to 10^8 cfu g^{-1}. Minimum detection times ranged from 5 to 17 hours and 4 to 14 hours, respectively, and the GLC measurement time itself was 3.5 minutes.[9] In routine use, only five to six injections were necessary to measure the minimum detection time for any sample. In other studies, the formation of hydrogen sulphide over aerobically stored meats may result from growth of Enterobacteriaceae and indicates a hygiene problem, while lactic bacteria may produce H_2S in vacuum-packed red meats.[23,59]

In some situations, GLC cannot only determine the presence of spoilage microbes in food products, but even partially differentiate them. Production of n-butyric acid and D(–) 2,3-butanediol served to differentiate sporeformers from nonsporeformers in cases of swelling of low-acid canned foods,[210] and the presence of butyric acid indicated clostridial contamination in commercially sterile vegetables, fruits, meats, fish, and dairy products.[68] Detection of n-butyrate helped establish the presence of *Clostridium estertheticum* and *C. laramie* in incidents of spoilage of vacuum packed raw beef.[40,46,47,118]

61.4 CONCLUDING REMARKS

The status and future for analytical food microbiology seem much brighter than they did even 10 years ago. Increased public concern with the microbial quality of foods, and the need for manufacturers to provide evidence to their customers that quality indices have been met, has spurred development of an enormous industry of kits and instruments. Whereas just a few years ago, detection of many pathogenic species required 5 or even 10 days, an equivalent analysis can usually be executed in 2 days, sometimes even in 1 day.

However, the value of "very fast" analyses to provide assurance of microbiological quality and safety is increasingly questioned. In a world of international food trade where safety is increasingly sought under the HACCP approach, it is unlikely that processes will ever be controlled by using tests for microorganisms; an exception would be foods where no other means of control is available, methods used for control are unknown, or foods are destined for sensitive consumers. Even if tests sufficiently rapid to permit corrective action to be taken online existed, the statistics of sampling plans practically means that the microbiologist's chances of reliably detecting defective (out of control) lots is very low.[104] (See also Chapter 60.) In HACCP-based industries, control over physical and chemical aspects of the microbial environment, for example, through using surface ATP measurement to indicate improper cleaning, or residual enzyme activity to indicate inadequate cooking, is likely to remain a better approach than attempting to detect the microbes themselves.

In the next few years, increasing use of technologies such as irradiation, flash-photolysis, and pressurization for controlling microbial pathogens in foods, may reduce even further the value of microbiological testing of foods as such. The future of microbiological testing probably lies in such areas as

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monitoring at the farm level (eg, to ensure control over the production of the animal and plant raw materials); in verifying control over environments of processing plants, distribution, and catering systems; and in providing compliance with regulatory requirements imposed on the industry, such as the mandated testing of carcasses for levels of *E. coli* and presence of *Salmonella* under the "Mega Reg" of the US Department of Agriculture.[261] Other major areas of application of microbiological testing will likely be in trouble-shooting problems occurring in the food industry and (despite the promise of HACCP) investigation of outbreaks of foodborne disease.

For some, but probably a few of these applications, tests should be as rapid as possible. Thus when sampling plant environments, it may be possible to collect the target organism with minimal interfering background material, which can be a great advantage to rapid analysis. At other times, however, the analyte will be in the presence of food, feces, or other problematic material, and the potential for very rapid tests can be expected to rely heavily on the availability of suitable techniques to separate and purify the analyte before analysis. In this respect, there is an urgent need to develop far more sensitive and reliable methods for detecting pathogenic viruses; these methods will almost certainly be based on nucleic acid amplifications methods. The current susceptibility of these techniques to interference by foods, and sanitizers emphasizes the need for more research into methods of separation and purification of these and other pathogenic agents.

In many other situations, extreme rapidity is not required. The centralized corporate control needed by organizations operating under ISO or total quality management (TQM) may be better served by methods capable of feeding data into information management systems. At the same time, however, there is a great potential for microbiological sampling and testing methods or devices with features such as low cost and high reliability, and being able to deliver reliable data when used by relatively unskilled personnel, perhaps even ensuring that the operator cannot sample the wrong location or miss an important spot. The tendency has been to direct the development of microbiological methods and testing equipment toward meeting the needs and wants of developed countries, as this is where the most profitable developments can be marketed. However, globalization of the world trade in foodstuffs and World Trade Organization agreements has led to the need to accommodate products from Third World countries where the degree of control expected in developed countries cannot be so easily be achieved at present because of the availability of resources and difficulty in controlling the food chain. This suggests the possibility of a huge potential market for devices more specifically developed for these regions.

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62

Detection of Microorganisms in Foods—Principles of Culture Methods

Roy Holbrook

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62.1 INTRODUCTION

Traditional methods for the detection, enumeration, and identification of microorganisms in foods depend to a large extent on using suitable culture media. To detect specific microorganisms, which very often only form a very small proportion of the total microorganisms present, selective media are used that enhance the growth of the target microorganism(s) (eg, pathogens) and suppress the growth of the rest. Diagnostic characteristics may be incorporated into such media, allowing recognition of the target microorganism(s) by a characteristic reaction such as a color change. Some foods exposed to freezing or mild heat treatments or preserved with chemical agents or by low water activity or low pH may contain microorganisms that are sublethally injured, and resuscitation conditions are then required before these bacteria can be reliably detected by growth in, or on, selective media. Other foods may develop a flora reflecting the preservation conditions and may require special media and practices for the detection of certain microorganisms,[61] for example, halophiles in highly salted fish products and osmophiles in foods preserved using sugar.

When testing food samples for the presence of general or specific groups of microorganisms, it is essential that only media and methods be used that have proven reliability and reproducibility in different laboratories, so that decisions made on the results of the analysis are assured.[49] Thus quality assurance (QA), the continued monitoring of working practices, equipment, and reagents, including the quality assessment of media, is essential, and extensive interlaboratory trials should be undertaken to demonstrate the value of both media and methods before they are adopted as standard practice or by official standards organizations. This chapter will highlight the essential general features of cultural methods and their use in the laboratory.

62.2 BACKGROUND

Many of the selective enrichment broths and differential agars used for detecting and isolating foodborne pathogens are based on clinical methods, adapted by food microbiologists to give the greater sensitivity and selectivity required for the microbiological analysis of foods. Microorganisms residing on humans and animals, be they pathogens,[69,122] commensals, or otherwise, generally are transient in the wider environment, and often exist outside their normal habitats in an attenuated or stressed physiological state.[143] In foodstuffs, such microorganisms may be further stressed by the use of preservation methods that are inhibitory to microbial growth, such as freezing or drying, but that may allow the survival of microorganisms in an attenuated form for long periods.[60]

To make a reliable and accurate analysis, the microbiologist must remove a representative sample from a food and transfer this sample to the laboratory so that the target microorganism(s) remains unchanged in numbers and characteristics. How well this task is performed reflects the statistical reliability of the sampling procedure (Chapter 60) and good laboratory practice that allows sufficient attention to the essential conditions required at all stages of analysis. These include media selection, formulation and preparation, sample storage and preparation for analysis, incubation conditions for resuscitation and growth, and appropriate isolation and recognition methods.

The range of methods used in food microbiology includes qualitative presence/absence tests, often used for pathogen detection, and quantitative methods used for the enumeration of viable cells of all types of microorganisms, and the appropriate use of nonselective broths or agars, recovery and/or selective enrichment broth, and/or differential or diagnostic agars. Tests may use a single medium or a sequence of liquid and solid media singly or in parallel.[171] Many of the analytical methods and media formulations that have become widely used in the microbiological examination of foods have been compiled into textbooks or manuals (eg, *Bacteriological Analytical Manual*;[50] *Compendium of Methods for the Microbiological Examination of Foods*;[2] *Foodborne Pathogens—An Illustrated Text*;[168] *Foodborne Microorganisms of Public Health Significance*;[7] *Microorganisms in Foods—Their Significance and Methods of Enumeration*;[79] *Practical Food Microbiology*;[141] and *Culture Media for Food Microbiology*.[30]) An extensive compilation of media for wider use has been published in the *CRC Handbook of Microbiological Media*.[6] Several hundreds of these formulations have been produced by commercial manufacturers as either dehydrated media or as ready-to-use media and are described in their company manuals.

62.3 PRINCIPLES OF MEDIA FORMULATION

The formulation of a new culture medium is greatly assisted by identifying, at the time of development, those attributes needed to improve performance over existing media used for the same purpose. This need may be the outcome of various observations, the suspicion that some strains or injured cells of the targeted species are not detected by existing liquid or solid media, that overgrowth by competitive flora masks the target organism, or that a better means to identify or speed organism recognition would be beneficial. From this it is possible to devise a strategy to achieve these goals. This could comprise consideration and determination of the following:

1. Scope of application and likely format of method
2. Identification of appropriate strains of target and competitor organisms
3. Determination of nutritional and other requirements for the optimal growth of undamaged cells and the recovery of likely damaged cells of the target microorganism
4. Determination of the optimum concentration, effective range, and the compatibility of likely selective and diagnostic agents to be incorporated in the medium
5. Establishment of optimum incubation conditions including temperature, time, and atmosphere
6. Assessment of performance against an established method(s) using a range of isolates from appropriate sources and using naturally contaminated foodstuffs
7. Validation of method by interlaboratory trials using comparative testing with a range of inoculated and naturally contaminated food and environmental samples.

62.4 CULTURE MEDIA COMPONENTS

Culture media are composed of a wide range of organic and inorganic components—some, such as the inorganic components, are chemically well-defined and of high purity and constant reproducibility, other components (mainly organic) are ill-defined, incompletely characterized, and with a variability that is poorly described or unknown.[22] Sources of nitrogen for growth may be ammonium ions, amino acids, peptones, or peptides; carbon as an energy source is most readily obtained from mono- and disaccharides; various inorganic salts, particularly phosphates, are added for buffering purposes and to aid metabolism together with Ca^{++}, Fe^{+++}, Mg^{++}, and Mn^{++}; and other trace metals commonly are present as impurities in the complex organic components. Some microorganisms need, or their growth is stimulated by, various vitamins, co-enzymes, and substances that reduce the toxic effect of oxygen radicals.[23,55] Some of these may be derived by the addition of whole blood, globin, serum, hemin, or egg yolk, compounds that are difficult to analyze but, being of direct biological origin, are of a more constant composition than the manufactured peptones and meat and yeast extracts. Extensive chemical analysis of complex organic components[128] prior to incorporation into media may fail to measure the critical components that allow optimum growth of specific microorganisms or production of toxins,[163] and growth studies with selected test strains may be the most effective means of identifying suitable batches of components. Many supplements to media are added at the time of use because they are unstable, have a short shelf life in solution, or are incompatible with the basic formulation. These components include antibiotics, egg-yolk emulsion, and blood components.

62.5 CHEMICALLY DEFINED MEDIA

Chemically defined media contain only constituents of known molecular structure and purity. They may be formulated as a special elective growth medium "which by restriction of nutrients and design of other components will allow the selective growth of the targeted organism(s) only,"[22] as the basis of a biochemical identification test or as a research tool in genetic or metabolic pathway studies. However, growth rate and microbial yield usually are lower than in complex media,[109] and few chemically defined media have gained wide application in food microbiology. An exception is minerals modified glutamate medium (MMGM), used for the detection of coliforms and *Escherichia coli*. It was introduced at a time when MacConkey broth was inconsistent in performance due to the variability of commercially available bile salts and peptone.[131] The medium has undergone extensive refinement and validation over 25 years, first used for the examination of drinking water[134] and later used as a resuscitation medium for the detection of *E. coli* in foods;[73,74] MMGM also allows the detection of stressed coliforms and *E. coli* surviving marginal chlorination in water.[133] The various modifications to MMGM are shown in Table 62–1 as an illustration of how a method may evolve over time.

62.6 NUTRITIONALLY COMPLEX MEDIA

Nutritionally complex media contain natural growth materials, the chemical composition of which is not completely defined.

62.6.1 Nutrient Components

Amino/Nitrogen Nutrients. Peptones and protein hydrolysates are the prime source of amino nitrogen in all complex culture media. They are produced by media manufacturers and specialist companies by industrial processes considerably modified from the original methods used in the early bacteriological media kitchens.[22] They are stabilized by concentration to pastes or powders and packaged to give a long shelf life. Manufacturers provide a range of peptones according to either the raw material used, the method of preparation, or by the blending of these.[159] They are sold under a range of product names.[23] Peptones may be broadly divided into the following groups.

- Peptones from animal tissues. Lean meat: peptic, tryptic, and pancreatic digests. Liver: papaic digest. Gelatin: pancreatic digest.
- Peptones from milk. Casein: hydrolysates prepared enzymatically by peptic, tryptic, or pancreatic enzymes or by acid treatment. Lactalbumin: pancreatic hydrolysates.
- Peptones from vegetable tissues. Soya meal: papaic digest, and products from groundnut and sunflower seed. All of these are rich in various natural carbohydrates unless removed by further processing.

All of these products differ in their peptide, peptone, and amino acid composition depending on their origin and the manufacturing process (Tables 62–2 and 62–3). Some products are formulated from mixed peptones to give a particular growth characteristic (eg, special peptone, proteose peptone, tryptose). Despite control exercised by the manufacturer, batch-to-batch variation can still affect optimal growth or maximal production of toxins.[163] The range of variation of the constituents of peptones is indicated in Tables 62–2 and 62–3.[22]

Table 62–1 Modifications to Folpers Medium To Formulate Minerals Modified Glutamate Agar

Formula g l^{-1}	*Folpers*[51]	*PHLS*[134]	*Gray*[62]	*Gray*[63]	*PHLS*[132]
Glucose	10	—	—	—	—
Lactose	—	10	10	10	10
Sodium formate	—	—	0.5	0.25	—
L(+) Glutamic acid	5.0	—	5.0	5.0	6.4
L(+) Arginine HCl	—	—	—	0.20	0.20
L(+) Aspartic acid	—	—	—	0.20	0.20
L(–) Cystine	—	—	—	0.02	—
Ammonium lactate	5.0	5.0	5.0	—	—
Thiamine	—	—	—	0.001	0.001
Nicotinic acid	—	—	—	0.001	0.001
Pantothenic acid	—	—	—	0.001	0.001
Magnesium sulphate $7H_2O$	—	—	—	0.20	0.10
Ammonium chloride	—	—	—	2.50	2.50
Ferric ammonium citrate	—	—	—	0.10	0.01
Calcium chloride $2H_2O$	—	—	—	0.20	0.01
K_2HPO_4	—	—	3.0	1.0	0.9
Bromocresol purple	0.01	0.01	0.01	0.01	0.01

PHLS, UK Public Health Laboratory Service

For the growth of some fastidious pathogens, certain peptones are beneficial or essential. For instance, an iron-rich liver digest peptone is incorporated into a *Campylobacter*-selective medium to enhance the growth and aerotolerance of fastidious *Campylobacter* species.[20,149] Tryptone is a rich source of tryptophan and is a component of media used to test the ability of bacteria to produce indole from tryptophan. Soya peptone is used in modified Rappaport-Vassiliadis broth where it enhances the growth of salmonella and maintains the pH of the prepared medium during storage.[165]

Table 62–2 Typical Amino Acid Concentrations (% w/w) in a Range of Oxoid Peptones, Hydrolysates, and Yeast Extract

	Peptones from:			
Amino Acids % w/w	*Meat*	*Soya*	*Milk*	*Yeast Extract*
Valine	2.09–4.11	3.65	2.89–5.52	1.0
Tyrosine	0.33–2.54	2.09	1.21–3.10	4.95
Tryptophan	0.25–1.35	0.64	<0.1–2.74	0.85
Threonine	1.25–2.75	1.68	1.37–4.32	2.73
Serine	0.83–3.61	0.67	0.96–1.53	3.42
Proline	2.38–12.10	3.40	3.58–9.08	0.88
Phenylalanine	1.71–4.66	0.38	1.78–3.60	3.78
Methionine	0.96–2.70	0.62	0.93–2.10	0.80
Lysine	3.89–6.02	3.77	2.62–9.65	5.40
Leucine	2.99–6.01	4.31	3.33–10.4	6.04
Isoleucine	1.02–4.58	2.51	1.20–4.99	4.81
Glycine	2.88–20.95	2.83	1.14–1.99	5.95
Glutamic acid	9.13–14.99	14.71	9.6–18.9	13.49
Cystine	0.12–1.66	0.53	0.22–2.41	0.76
Aspartic acid	4.68–9.4	7.06	2.71–7.55	7.07
Arginine	2.70–7.75	4.64	0.75–5.80	3.31
Alanine	3.40–6.75	2.57	1.73–4.47	0.91

Source: Data from E.Y. Bridson, *The Development, Manufacture and Control of Microbiological Culture Media*, © 1994, Unipath Ltd.

Table 62–3 Range of Typical Concentrations of Elements (ppm) and Other Components in Oxoid Peptones, Milk Peptones, and Hydrolysates and Yeast Extract

	Peptones from			
Substance	*Meats*	*Soya*	*Milk*	*Yeast extract*
Co	0.1–0.7	0.2	<0.1	<0.1–3.1
Zn	3.9–127	12	4.1–42	1.2–94
Sn	0.7–8.3	1.1	1.5–14	3.0–10.5
Mn	0.5–6.0	1.0	0.5–28	0.4–1.7
Pb	0.1–0.8	0.3	0.1–1.4	0.3–0.7
Cu	1.0–60	2.0	<1–3	2.0–3.0
Fe	12–190	90	3.0–54	9.0–52
Mg	94–845	1530	24–1,196	140–703
Ca	98–885	225	86–11,000	140–153
% K	0.04–3.6	3.3	0.07–2.4	0.5–7.0
% NaCl	1.0–9.3	0.4	0.3–30.2	0.3–1.1
PH	5.4–7.2	7.2	6.5–13.3	5.6–7.2
% total N	9.5–15.9	9.1	5.3–13.3	1.1–13.3
% amino N	2.2–3.5	2.3	1.8–5.4	0.6–5.3
Ash	4.2–16	13.5	6.0–37.3	1.2–13.3
Moisture %	<5	5.8	<5	<5

ppm, parts per million

Source: Data from E.Y. Bridson, *The Development, Manufacture and Control of Microbiological Culture Media*, © 1994, Unipath, Ltd.

Extracts. Aqueous extracts of animal, plant, or yeast cells contain less organic nitrogen than the acid or enzyme extraction methods and, therefore, sustain less microbial growth when used alone. However, importantly, they contain many growth factors destroyed by the manufacturing process used for the production of peptones. Extracts are therefore often used to complement the peptones and hydrolysates in media formulations.

Meat extract originally was produced in the laboratory media kitchen by the cold infusion of lean, fat-free meat such as beef heart,[176] then commercially as a paste by concentration of the extract to >20% solids and now as a more refined, sugar-free, dried powder. The composition of the meat extract depends on the meat source time and temperature of extraction.[13] Meat extracts contain amino acids, peptides, purines, organic acids, urea, ammonium salts, and coloring matter and are highly buffered: they are also rich in B vitamins but low in thiamine.[13,159,176]

Yeast extract is a widely used nutrient component in culture media, being composed of amino acids, peptides, water soluble vitamins (including thiamine), nucleic acid residues, and the carbohydrates glycogen, trehalose, and glucose (Tables 62–2 and 62–3). Bacteriological yeast extract is prepared by the lysis of washed cells of *Saccharomyces* species using a mechanical means and slowly raising the temperature to 50° C; isopropyl alcohol may be added to improve lysis. The cell-free extract is then pasteurized to destroy the enzymes present and rapidly concentrated either to a paste or a spray-dried powder; Bridson and Brecker demonstrated that yeast extract derived from Bakers' yeast gave superior growth characteristics to that from Brewers' yeast, reflecting the original growth media used in the production of these yeasts.[23]

Malt extract, the water-soluble wort of malted barley, is used for the growth of yeast and molds. Its final composition will vary according to the species of barley used, its condition at harvest, and the conditions at the time of fermentation. Malt extract has a pH of about 5.5 and is rich in sugars and amino-compounds (Tables 62–2 and 62–3). A comparison of the vitamin content of meat, malt, and yeast extracts is given in Table 62–4.

62.6.2 Carbohydrates

Many media formulations contain added glucose at concentrations of 1% or less to supply a readily usable energy source. Other media contain a specific carbohydrate that, through its use, acts as an indicator for a particular group of organisms, such as lac-

Table 62–4 Vitamin Content of Meat, Yeast, and Malt Extracts (μg g^{-1})

	Meat Extract			
Vitamin	*Fresh Extract*	*Commercial Product*	*Yeast Extract*	*Malt Extract*
Cyanocobalamin	0.3–1.0	0.2	0.01	*
Thiamin (B_1)	0.9–3.0	0–10	20–70	24
Riboflavin (B_2)	1.8–3.5	30–35	55–100	25
Nicotinic acid	24–102	1,000–1,200	*	0.7
Pyridoxine (B_6)	0.77–4.0	5	25–35	44.5
Pantothenic acid	4.9–15	25	20–100	31.5
Choline	760	1,500	1,000–2,000	*
Folic acid	*	*	30–40	5.5
Biotin	*	*	0.5–1.0	0.48
Inositol	*	*	1,000–1,700	2.5

* Value not given

Source: Data from E.Y. Bridson, *The Development, Manufacture and Control of Microbiological Culture Media*, © 1994, Unipath Ltd.

tose in the presence of bile by coliform organisms in MacConkey broth or on violet red bile agar. Some media contain a mixture of carbohydrates such as lactose and sucrose in phenol red brilliant green agar to encompass either greater differentiation between targeted and nontargeted organisms or to increase the range of bacteria targeted. The ability of microorganisms to use a carbohydrate may also be used as a means of identification (eg, mannitol by *Staphylococcus aureus* in Vogel-Johnson agar). An array of individual carbohydrates, each in a nutritionally simple base medium or agar together with a pH indicator (eg, purple agar base), is used as part of a battery of biochemical tests to differentiate one species from another.[11] To aid in the repair of some bacteria and stimulate growth of others, pyruvate replaces a carbohydrate source, as in Baird-Parker agar,[8,75] and some media such as Cefsulodin Irgasan Novobiocin agar[126] or pyruvate egg yolk mannitol bromothymol blue agar (PEMBA)[72] contain both pyruvate for repair and mannitol for differentiation. Pyruvate may act as a carbon source or as a means to neutralize superoxide radicals and thereby reduce the toxicity of media, particularly to injured cells with damaged membranes.[25,55] In many media, no carbohydrates are added and the small amounts of carbohydrates present in other components are readily used by the organism to provide energy. For example, the growth of a *S. aureus* in heart infusion broth first uses carbohydrates present from its meat infusion, causing a fall in pH, and when these carbohydrates are exhausted the pH increases as a result of decarboxylation of amino acids. Where carbohydrates present in media are not used as an indicator of metabolism, then some degree of buffering to maintain the pH at the optimum for growth or selective action is necessary.

62.6.3 Buffers and pH Indicators

Some buffering results from the presence of peptones and amino acids, but when carbohydrates are included in a medium, this buffering capacity is insufficient to maintain an optimal pH. An effective buffering system is one that has maximum buffering capacity at the desired pH. This maximum is around the pK_a of either the weak acid or base component of the buffer. Thus for neutral media at pH between 6.8 and 7.2, hydrogen phosphate and dihydrogen phosphate ($pK_a = 7.21$) as either sodium or potassium salts are commonly used. In an acidic medium such as lactobacillus sorbic acid agar, with a pH of 5.0, a citrate/acetate buffer is used. An alternative means of maintaining neutrality (eg, in tetrathionate broth) is to use chalk to neutralize the weak acids formed from bacterial metabolism. Indicators of pH change are selected so that their color change corresponds to the change in pH of the medium. Those in common use in bacteriological media are neutral red with change in color from red to amber through pH 6.9 to 8.0; phenol red with change in color from yellow to red through pH 6.8 to 8.4; bromothymol blue changing from yellow to blue through pH 6.9 to 8.0; bromocresol purple changing from yellow to violet through pH 5.3 to 6.8.

Phosphates in combination with glucose in media can cause the accumulation of hydrogen peroxide in autoclaved media exposed to oxygen, resulting in possible increased toxicity to injured microorganisms.[23]

62.7 SELECTIVE AGENTS

A wide range of chemical substances is used to generate the selective conditions that will favor the growth of one species or a related group of microorganisms and/or suppress the growth of other microorganisms present in a culture medium. These selective agents include antibiotics, dyes, surface-active agents, and inorganic and organic chemicals variously having a wide or more specific inhibitory action. Each agent may be used alone or in combination. The selective action may be achieved by one or several of the following means:

Inhibition of Competitors. Bactericidal action may cause immediate or progressive decline in numbers. Bacteriostatic action may ensure little or no increase in the original numbers of competitors and a probable slow decline in their viability.

Differences in Lag Time. Some targeted microorganism(s) have a shorter lag time in the selective conditions and thus outgrow other organisms.

Change in Relative Growth Rate. If the targeted microorganism has a shorter generation time than its competitors in the selective conditions, an increase in its relative numbers results.

Incubation Conditions. Use of an appropriate incubation temperature can enhance the growth of a targeted microorganism, such as growth at 55° C for the selection of thermophiles. Use of an optimum incubation atmosphere can enable selection of the targeted microorganism (ie, aerobes, strict or less-sensitive anaerobes, microaerophilic species).

Use of the ideal selective conditions would achieve the inhibition of the most robust competitors while allowing good growth of the most sensitive targeted microorganism; however, the probability of a formulation achieving this balance is, at best, very rare. Thus in liquid selective media, the targeted microorganisms may dominate all other species present or may only form a small proportion of the total flora. On solid selective media, the targeted microorganism should grow well, while competitors grow less well, are partially inhibited and give rise to microcolonies, remain viable but fail to divide, or are killed. Where microcolonies are formed or cells remain viable, these may inadvertently be picked from the medium together with the targeted colony when subculturing. Here, direct streaking onto a nonselective agar medium rather than inoculation into a liquid medium is necessary to recognize whether the subculture is mixed and to allow the isolation of a pure culture. Many selective agents, while not inhibitory to the growth of robust strains of the targeted organism, are inhibitory to these same strains if stressed or damaged. Mossell and van Netton have reviewed the harmful effects of selective agents in culture media that are inhibitory to the recovery of injured foodborne microorganisms.[115]

Antibiotics. These are the most widely used selective agents because of the numbers available, and the differing spectra of their antimicrobial activity. Examples of those used in food microbiology are listed in Table 62–5.

Where antibiotic solutions are prepared in the laboratory from dry powders, it is essential that the antimicrobial activity of the antibiotic is known, and that the amount weighed out is adjusted accordingly so that the intended concentration is used.

Dyes. Prior to the advent of antibiotics, dyes were studied extensively for their antimicrobial properties and their use in clinical medicine and in the laboratory. The triphenylmethane dyes were the most effective in culture media and are still used in food microbiology to inhibit Gram-positive bacteria.[52] Some dyes show some differential activity within the Gram-positive species.[54] The acridine dyes, of which acriflavine (a variable mixture of 3,6-diaminoacridine dihydrochloride and 3,6-diamino-10-methylacridinium chloride) are the most commonly used, are very inhibitory to injured bacteria and mutagenic to others.[1] Brilliant green is inactivated by heat, bile salts, and light; therefore, appropriate conditions in the preparation, storage, and use of media should be implemented.[9,67,164] As the chemical composition, purity, and therefore antibacterial activity of dyes can be variable, checks with appropriately sensitive test strains are essential in quality assessment of media.[121]

Surface-Active Agents. Three bile products are widely used in selective media. These are: ox bile, a crude dried preparation of variable composition; bile salts, a partially purified material obtained by acid precipitation that separates the bile salts from the bile pigments and other components in the crude bile; and bile salts No. 3, the most refined material, containing specially modified fractions of the bile acids. Compared with ox bile, the bile from other animals will show differences in selectivity. Other refined products from crude bile such as desoxycholate and taurocholate also are used as inhibitors of Gram-positive bacteria in enteric media. Because of the biological variation in crude bile and also differences in activity of the manufactured bile products, sodium lauryl sulphate may be used as an

Table 62–5 Antibiotics Used in Some Microbiological Media for the Analysis of Foods

Antibiotic	*Medium Formulation*	*Bacterial Species*
Acriflavin HCl	Fraser broth	*Listeria* species
	Buffered listeria enrichment broth	*Listeria* species
	PALCAM agar	*Listeria* species
	Lovett listeria broth	*Listeria* species
Amphotericin	Modified charcoal cefaperazone desoxycholate broth	*Campylobacter* species
Ampicillin	Tryptone soy ampicillin agar	*Aeromonas hydrophila*
	Tryptone soy ampicillin broth	*Aeromonas hydrophila*
Cefaperazone	Modified charcoal cefaperazone desoxycholate broth	*Campylobacter* species
Ceftazidime	PALCAM agar	*Listeria* species
Cefixime	Cefixime tellurite MacConkey agar	*Escherichia coli* O157
Cefotetan	Oxford agar	*Listeria* species
Cefsulodin	Cefsulodin irgasan novobiocin agar	*Yersinia enterocolitica*
	BPW + VCC	*Escherichia coli* O157
Chloramphenicol	Chloramphenicol malt extract agar	Yeasts and molds
	Chloramphenicol glucose agar	Yeasts
Colistin sulphate	Oxford agar	*Listeria* species
	MOX listeria broth	*Listeria* species
Cycloheximide	Lovett listeria broth	*Listeria* species
	Buffered listeria broth	*Listeria* species
	Oxford agar	*Listeria* species
	PEMBA	*Bacillus cereus*
D-cycloserine	Tryptose sulphite cycloserine agar	*Clostridium perfringens*
Fosfomycin	Oxford agar	*Listeria* species
Moxalactam	MOX listeria broth	*Listeria* species
Nalidixic acid	Fraser broth	*Listeria* species
	UVM Listeria broth	*Listeria* species
	Lovett Listeria broth	*Listeria* species
Novobiocin	Tryptone soy broth + novobiocin	*Escherichia coli* O157
	Cefsulodin irgasan novobiocin agar	*Yersinia enterocolitica*
	Shigella novobiocin broth	*Shigella* species
	MacConkey novobiocin broth	*Shigella* species
	Hecktoen enteric novobiocin agar	*Shigella* species
Oleandomycin	Oleandomycin polymixin agar	*Clostridium perfringens*

continues

Table 62–5 continued

Antibiotic	*Medium Formulation*	*Bacterial Species*
Oxytetracycline	Oxytetracycline glucose agar Oxytetracycline malt extract agar	Yeasts and molds Yeasts and molds
Polymyxin B	PALCAM agar Salt polymyxin agar Mannitol egg yolk polymyxin agar Oleandomycin polymyxin agar	*Listeria* species *Vibrio parahaemolyticus* *Bacillus cereus* *Clostridium perfringens*
Streptomycin sulphate	Streptomycin thallium acetate agar	*Brochothrix thermosphactam*
Sulphadiazine	Oleandomycin polymyxin agar	*Yersinia enterocolitica*
Sulphamezathine	Baird-Parker agar	*Staphylococcus aureus*
Ticarcillin	Irgasan ticarcillin agar	*Yersinia enterocolitica*

BPW, Buffered Peptone Water; *MOX*, Moxalactam Colistin Listeria Broth; *VCC*, Vancomycin Cefsulodin Cefixim; *UVM*, University of Vermont Media
Note: This is not an exhaustive list.

alternative surface-active agent to suppress the growth of Gram-positive bacteria; it also has the advantage of a defined chemical composition that gives a more consistent antimicrobial activity.

Inorganic Chemicals. These include tetrathionate and selenite, used as selective agents in media for salmonellas; potassium tellurite and lithium chloride used to inhibit Gram-negative species in Baird-Parker agar and other media; dichloran (2,6 dichloro-4-nitroanaline) used in mycological media to inhibit bacteria and slow the spreading of colonies of fungi; irgasan, a disinfectant, used as a selective agent in media for the isolation of *Yersinia* species; thallous acetate used as a the selective agent in thallous acetate tetrazolium glucose agar for the differentiation of *Enterococcus faecalis* from *Enterococcus faecium*; and cetrimide (cetylmethyl ammonium bromide) in cephaloridine fucidin cetrimide agar for isolating *Pseudomonas* species.

62.8 DILUENTS

For the preparation of suspensions and dilutions of foodstuffs, 0.1% peptone water is most commonly used. However, when requiring a neutral suspension, the resulting mixture may require pH adjustment when used to prepare suspensions of acidic foods. The use of buffered peptone water for such products often can avoid this pH adjustment; the risk of extraneous contamination during pH adjustment is also prevented. Peptone, in the absence of significant amounts of other organic matter, also neutralizes the effect of any low levels of inhibitory metals that may originate from external sources, such as water or glass containers.[92] In a study of the survival of *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Salmonella typhimurium* in six diluents during their storage for 2 hours at room temperature, five of the diluents were bactericidal to one or more test strains, the sixth diluent, 0.1% peptone water, caused little change in any of the viable counts.[95] In a further study of the effect of seven diluents on the viability of yeasts isolated from foods, 0.1% yeast extract and 0.1% malt extract allowed the greatest survival and distilled water and 0.85% saline the least.[104] The salt-tolerant yeast *Debaryomyces hansenii*[3] and xerophilic yeasts (eg, *Zygosaccharomyces* species, *Schizosaccharomyces pombe)* required osmotic protection (eg, use of 20% to 30% glycerol in peptone water) when serially diluted prior to plating.[16] While fungal spores appear unaffected by diluents and 0.1% peptone water generally is regarded as satisfactory, the optimum conditions for handling fungal hyphae are not well described.[130]

For presence/absence tests for specific microorganisms in foods, it is common practice to dilute samples directly into the growth medium and to homogenize the mixture. Certain foods need specific treatments to make a well-dispersed suspension. For example, chocolate requires warming at 45° C until melted, and samples rich in fat require the addition of small quantities of sterile Triton X-100 or Tween 80. When preparing dried materials for the examination of Gram-negative pathogens by presence/absence tests, controlled rehydration to minimize osmotic shock to damaged cells is beneficial to their recovery and detection. Two methods to achieve this have been described: by carefully layering the dried sample onto the surface of the liquid so that the sample slowly absorbs water for up to 30 minutes before homogenization or the stepwise addition of diluent, starting with a 1 to 1 w/v ratio and progressing to a final 1:10 ratio through about 30 minutes.[167]

To neutralize any antimicrobial agents that may be present on food processing equipment after sanitation, swabs used to check the effectiveness of sanitation should be placed in universal quenching agent (0.1% peptone, 0.1% sodium thiosulphate, 0.5% Tween 80, and 0.7% lecithin) prior to plating onto microbiological media.

62.9 MICROBIAL GROWTH MEDIA

As well as classification by composition, growth media may also be characterized according to use; such classification will be used in this section of the chapter.

62.9.1 Liquid Media

General Purpose Nonselective Broths. These comprise broths having no selective or differential features, such as nutrient broth or heart infusion broth. These are used for general tasks such as the growth of microorganisms to obtain pure cultures or to produce inocula for further testing, such as species identification or strain characterization. Nonselective broths also have been formulated for the growth of specific groups of organisms that benefit from the presence of particular growth substrates, for example all-purpose Tween broth for the growth of lactic acid bacteria,[138] TSB (tryptone soy broth) for the growth of yeasts, and Robertson's cooked meat broth[142] for the growth of microaerophilic or anaerobic microorganisms. These media can be used for the short-term maintenance of a culture for periods of about 1 week for many, but not all, bacteria. While technically simple, repeated subculture in broth has a risk of contamination and an undetected change in strain characteristics. For the characterization of an organism, it is preferable to grow the inoculum in a complex medium rather than a defined medium because the former will lead to the better expression of the biochemical and morphological characteristics of the organism.[28]

Pre-enrichment Broths. Pre-enrichment media are nonselective media formulated to allow the repair of any sublethally injured microorganisms present prior to their exposure to the selective agents in selective enrichment broths.[44] Direct inoculation of injured cells into selective media can result in the death of some or all of them, the latter giving a false-negative result. Some selective broths are formulated to overcome injury, as is Giolitti-Cantoni broth for the detection of low numbers of *Staphylococcus aureus* in foods.[57] Suitable pre-enrichment conditions, in combination with productive selective enrichments, are essential for the reliable detection of low numbers of foodborne pathogens in foodstuffs. Much of the investigative work done on the advantages of various formulations of pre-enrichment broths (Table 62–6) concerns their use for detecting low numbers of salmonella in raw materials for processed foods or final products.

While the principles involved are described here in relation to salmonella, they also apply to other injured, pathogenic bacteria. During the incubation of a pre-enrichment culture of a food sample, injured and uninjured cells of salmonellas must recover, and then multiply to numbers that ensure both their transfer on subculture to a selective medium and their survival in the selective medium in sufficient numbers that their growth is ensured. At the end of pre-enrichment incubation, cells must not be in any way attenuated or they may be sensitive to the selective agents in the selective medium. A nonselective pre-enrichment culture will allow the growth of the majority of the microorganisms present in a food sample; these microorganisms may overgrow the target microorganism. By the time repair has occurred, other uninjured or less-injured microorganisms may form the dominant species. When the dominant species enter their own stationary phase, they may inhibit the further growth of all other species present, including the targeted species that may still be in such small numbers that their detection after the selective enrichment stage is uncertain (see the pre-enrichment stage in Figure 62–1). This stationary phase inhibition effect was first described by Jameson[89] and is discussed in detail later.

The use of short pre-enrichment incubation (eg, 6 to 8 hours), while permitting same-day subculture, is not sufficient to allow the recovery and multipli-

Table 62–6 Development and Use of Pre-enrichment Broths for the Isolation of *Salmonella* Species in Foods and Animal Feeds

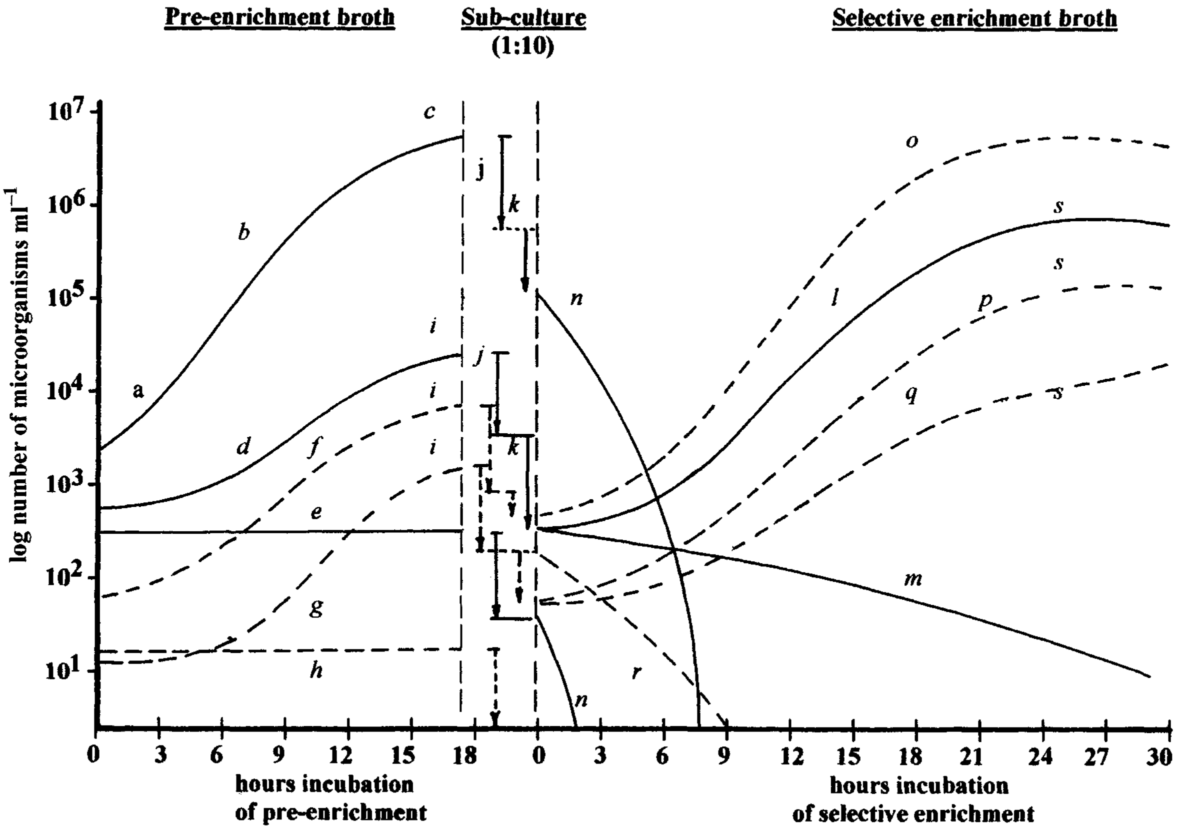
Date	*Medium*	*Purpose*	*Comments*	*Reference*
1955	Nutrient broth	Recovery of *S. paratyphi* from flour	>10-fold increase in MPN estimates	Thompson[161]
1961	Lactose broth	Dried foods and egg products	Improved detection rate	North[120]
1963	Nutrient broth	Egg and egg products	Improved detection rates	Hobbs & Alison[70]
1972	Buffered peptone water	All dry foods, processed foods, animal feeds, meats	Resuscitation of stressed cells	van Schothorst & van Leusden[166]
1974	Buffered peptone water	Minced meat and animal feed	Resuscitation of stressed cells	Edel & Kampelmacher[44] and ISO 6579[86]
1977	Lactose broth and peptone water	Frozen sweet corn	Study on maintenance of pH during incubation	Sadovski[144]
1977	Lactose broth	Recommended for general use (eg, milk and milk products, dry powdered food mixes, meats, fish, dried vegetables and fruits)	Fall in pH to 5.5–5.0 from lactose use selects for salmonella. Below pH 5.0 becomes inhibitory.	BAM 1997[50]
1978	Eugen broth, Clausen broth, Tergitol broth, EE broth, GN broth	Wide range of foods of low- and high-moisture content; animal feeding stuffs	Study to shorten incubation times	D'Aoust & Maishment[36]
1992	Distilled water + brilliant green	Dry whole milk, nonfat dry milk	Inhibition of Gram-positive flora in the reconstituted milk used as growth medium	BAM 1997[50]
1992	Trypticase soy broth	Whole eggs, spices, herbs	Neutralization of natural inhibitors in the test material	BAM 1997[50]
1992	Sterile nonfat dried milk	Candy and chocolate	Neutralization of natural inhibitors in the test material	BAM 1997[50]
1992	Trypticase soy broth + 0.5% K_2SO_4	Onion and garlic flake and powders at 1:10; allspice, cloves, cinnamon, and oregano at ≤ 1;100 dilution	Neutralization of natural inhibitors in the test material	BAM 1997[50]
1998	Buffered peptone water + enzymes	Digestion of gelling agents—starch, gums, and celluloses	Also in combination with 1:100 dilution	ISO 6579[86]

cation of all injured cells.[9,36,100,101] A general optimum time for conventional pre-enrichment incubation is 16 to 20 hours for salmonellas. This time is a compromise between a longer incubation time that may allow better recovery of severely damaged salmonellas, and the increased risk of decline in the physiological status of undamaged salmonellas and/or gross overgrowth by the competing bacteria in heavily contaminated samples. Where dilutions of a food material greater than 1 in 10 are necessary, to overcome, for example, naturally occurring inhibitory agents,[50] then a 20 hours to 24 hours incubation is recommended.

The use of chemically defined media may enable increased recovery of injured salmonellae, previously grown under minimum nutrient conditions, compared with those grown under rich nutrient conditions. This was demonstrated by the better recovery of heat-injured *Salmonella senftenberg* in chemically defined M9 medium than in nutrient broth and lactose broth with or without the addition of food samples.[175] The significance of this to the isolation of salmonella from foodstuffs is questionable because it is most likely that salmonella present in a food sample will be derived from a nutrient-rich environment, although the physiological status of the cell will be unknown depending on its past history. However, buffered peptone water, which is nutritionally less rich than nutrient or lactose broth, has been shown to be more effective for the repair of salmonella.[153]

Some injured salmonellae have prolonged repair times.[100] Using an automated growth analyzer, a device based on optical density measurement and able to study the growth from single-cell inocula in liquid media, Stephens et al.[153] demonstrated that the estimated lag (repair) times of heat-injured *S. typhimurium* cells varied from about 6 hours to >30 hours in buffered peptone water, TSB, lactose broth, and heart infusion broth. This effect was seen using different manufacturers' media and different batches of the same media from one manufacturer. As the number of injured cells added to each test unit increased above 1 cell, the apparent lag time decreased, being indicative of the growth of the least-damaged cells in each subpopulation. With a larger inoculum (ie, 10^2 to 10^3 cells) of injured cells, lag times similar to those determined by traditional methods from growth curves studies were obtained.[153] Evaluation of batches of peptone permitted the selection of the best product for the recovery of the injured cells. The variable lag time of individually injured salmonella cells subsequently was reduced by the addition of oxyrase® to the medium. This removed the toxic oxygen molecules in the medium and reduced the oxidative stress on the injured cells [Stephens, in press]. These findings have significant implications for the use of pre-enrichment and for our understanding of the performance of current methods to reliably recover one or a few (<5) injured salmonella in the most demanding test situations. Extended lag times also were found for attenuated *Listeria monocytogenes*[112] and *Escherichia coli* O157 [Stephens, in press]. Thus the individual cells of severely stressed Gram-positive or Gram-negative bacteria either require longer incubation times for repair than is currently practiced or re-formulation of the growth medium to give shorter recovery times.

Selective Enrichment Broths. Selective enrichment broths, with or without prior pre-enrichment, may be used as a single presence/absence test, or in a dilution series to estimate low numbers of targeted species using multiple tubes of media in a most probable number format.[119] In a liquid enrichment broth inoculated with a mixture of microorganisms, competitive growth occurs between all species able to tolerate and use the selective conditions. Rarely from a heterogeneously contaminated sample does any selective enrichment allow the growth of the targeted species alone.[93,125] Enrichment broths may be inoculated with either a food sample, a pre-enrichment broth, an environmental swab, a filtered water sample, or from a primary enrichment to give a further, secondary enrichment. In all these cases the targeted microorganisms generally comprise only a very small proportion of all the bacterial species present.[125] In some highly selective media, the inoculum of healthy cells of the targeted species may decline during adaptation to the new medium.[78,164,165] The selected microorganisms then grow to give a culture of unpredictable composition when the stationary phase is reached. It is therefore understandable that the performance of a formulation varies. Ideally the targeted microorganism will ultimately form the dominant bacterial species in the enrichment broth, but in most cases this is not so. Uninhibited competitors may also increase to high numbers either initially or as a result of the growth of the targeted species, changing the selective conditions of the medium. The effectiveness of each selective agent in a selective broth is influenced by any synergistic or antagonistic effects between the selective agents, especially antibiotics,[20,21] and by the components of the food sample diminishing their effectiveness.[148] Where alternative enrichment broths based on different selective agents are used in parallel, then where one medium fails to inhibit a dominant competitor another enrichment may be effective, so that



Events in pre-enrichment media during 18 hours incubation
a—lag period as measured by the total viable count
b—exponential phase of dominant microorganisms
c—initiation of stationary phase in the dominant species
d—growth curve of a nontarget species having a longer lag phase
e—nontarget species unable to grow but survives in the growth medium
f—targeted bacteria—growth curve from small numbers
g—targeted bacteria—growth curve of strain having a prolonged lag time
h—prolonged lag time of severely damaged target microorganism; doubtful detection
i—the Jameson effect—lesser populations passing into premature stationary phase as a result of (c)

Events occurring on subculture of pre-enrichment to selective enrichment broth
j—dilution of population (1/10, 1/00, or other)
k—variable degrees of death that occur with nontarget microorganisms on inoculation into the selective enrichment broth and possible decrease in numbers of some sensitive strains of the target organisms

Events during incubation of selective enrichment broth
The data assume that the targeted bacterium forms a minor part of the total population from the pre-enrichment broth
l—growth curve of a competitive species not inhibited by selective agents and part of the dominant flora of the pre-enrichment broth
m—decline of competitors unable to grow (bacteriostatic effect)
n—death of competitors (bactericidal effect)
o—growth curve of a robust target microorganism with shortest lag and generation time to form dominant species within 24 hours incubation
p—growth of a less vigorous target strain that forms a smaller proportion of the total population and may not be isolated when cultured onto selective agars at 24 hours
q—A poorly growing target strain that will not be isolated at 24 hours and numbers will increase during secondary phase enrichment
r—Target strain sensitive to a component of the selective enrichment (eg, brilliant green)
s—Jameson effect—the inhibition of other strains as the dominant strain enters its own stationary phase in the enrichment broth

Figure 62–1 Schematic Representation of the Range of Events That Can Occur during Pre-enrichment and Selective Enrichment Culture

Table 62–7 Isolation of Salmonella from 51 Samples of Liquid Whole Egg

	Salmonella Isolated after the Elapsed Incubation Times Stated						
Sample Number	*Pre-enrichment in Nutrient Broth and Subculture at 24 h and 48 h:*				*1st Tetrathionate broth*		*2nd Tetrathionate*
	24 h	*48 h*	*72 h*	*96 h*	*48 h*	*72 h*	*72 h*
1	-	-	-	-	-	-	++
2	-	-	-	-	+++	+++	+++
10	-	-	-	-	-	++	-
14	-	-	-	-	+	++	-
17	-	-	-	-	-	-	-
30	+	-	-	-	+++	+++	-
43	++	+	+	-	+++	+++	+++
44	-	-	-	-	+++	++	+++

There were 8 positive results obtained using pre-enrichment in nutrient broth and streaking, followed by selective enrichment for 24 hours and 48 hours in tetrathionate broth and further subculture at 24 hours into a further tetrathionate broth. All broths streaked onto bismuth sulphite agar.

Source: Data from J.E. Jameson, A Discussion of the Dynamics of Salmonella Enrichment, *Journal of Hygiene (Cambridge)*, Vol. 60, pp. 193–207, © 1961.

the target species is subsequently detected.[37] Jameson[89] studied the interaction of proportions of different Enterobacteriaceae species in a salmonella-selective broth. Provided there were no antagonistic effects between the species, all were found to grow initially with very similar lag and generation times as when growing alone in that medium. When the culture approached the stationary phase, primarily due to the numbers of the dominant species, then cell division of all other species present simultaneously declined (Figure 62–1). This was irrespective of the stage at which the less numerous species were in their own growth cycle. Jameson described this as the primary stage of enrichment. It was subsequently followed, on extended incubation, by a secondary stage in which a succession of less numerous species grew to form a larger part of the total population. This secondary stage of multiplication was at rates considerably slower than those in the first stage. Even different strains of the same species (eg, *Escherichia coli* or *Salmonella*[68]) when grown together demonstrated this Jameson effect. Figure 62–1 depicts the various events that can occur in the use of pre-enrichment followed by selective enrichment.

Thus, cultures containing salmonella and plated onto selective agar media after 24, 48, 72, and 96 hours of incubation may be positive on one occasion but negative on another, or salmonellae may be isolated on all occasions (Table 62–7). This interactive effect was dependent on the initial numbers of salmonella and competitors present.[89] Park and Sanders also recommended that enrichment cultures used for the detection of *Campylobacter* species should continue for up to 96 hours to improve isolation rates.[123] Such culture dynamics probably contribute to the performance of cold enrichment previously used for the enrichment of *Yersinia* species[113,146] and *Listeria*.[99]

Some selective enrichment media now recommended for the detection of certain foodborne bacteria are very productive following continuous refinement of formulae over many years (eg, Rappaport-Vassiladis broth for *Salmonella*[169] and tryptose-sulphite-cycloserine agar for enumerating *Clostridium perfringens*[117]).

The selective action of some enrichment broths is not sufficiently good, however, to allow recovery of all targeted cells following pre-enrichment or the direct enrichment of heavily contaminated samples and, for these, other strategies have been developed. For example, in the detection of *Listeria* species a reduced level of selective agents in the primary selective medium is used in the International Standards Organization (ISO) and US Department of Agriculture Food Safety Inspection Service (USDA-FSIS) methods.[87,110] This allows recovery and growth of most listeria (severely injured cells may not recover), while having some selective action to minimize competition. A second enrichment broth is then inoculated to increase numbers to levels that are detectable when plated onto selective agars.

Another strategy is the combined use of two or three selective media based on different selective principles. Thus tetrathionate,[93] selenite,[97] or malachite green and pH[166] are widely used for salmonella isolation.[86] Where one medium fails to give detectable numbers of salmonella, another often succeeds.[67] Two enrichment broths are used in parallel in each of the ISO Standards for the detection of *Vibrio parahaemolyticus*,[82] *Yersinia enterocolitica*,[83] thermotolerant *Campylobacter* species,[85] and *L. monocytogenes*.[87]

A further enrichment culture strategy is the sequential addition of selective agents to permit resuscitation, then selection of target species. This principle was described for the isolation of *Campylobacter jejuni* and *C. coli*.[123,124] In the initial broth, pyruvate is used to aid recovery, and vancomycin and trimethoprim lactate are used to give some selectivity. After incubation at 32° C for 4 hours under microaerophilic conditions (by sparging with 5%O_2:10%CO_2:85%N_2), further antibiotics, cefoporazone, and cycloheximide are added and incubation is continued at 37° C for a further 2 hours before the temperature is raised to 42° C for 40 to 42 hours to increase selectivity prior to plating. Thus recovery of *Campylobacter* spp. and their exposure to the selective agents are progressive events. The US Food and Drug Administration's *Bacteriological Analytical Manual* method for isolating listeria adopted a similar technique with resuscitation in a pyruvate-containing medium before the addition of antibiotics.[50]

Secondary enrichment (not to be confused with secondary-phase enrichment described by Jameson[89]) may be used to advantage when primary selective enrichment fails to allow isolation of the target organism due to heavy overgrowth by competitors. Here the first enrichment is subcultured into a second enrichment broth and that is plated out after further incubation. Greater success may result when the second enrichment is based on different selective principles from the first enrichment. Thus improved performance and reliability result from a strategy that optimizes recovery of the most stressed, but viable, target cells followed by the imposition of selective conditions as soon as recovery will allow, and before overgrowth by competitors occurs. How well the new systems of timed release[154] of selective agents, following the enhanced repair rates devised by Stephens et al.,[153] will further improve the recovery of damaged and undamaged microbial cells from foods remains to be seen.

Immunomagnetic Separation. Where a targeted species has a specific antigen(s), immunomagnetic separation (IMS) is a useful means of separating it from competitors and food materials allowing various techniques to be used to identify the separated microorganisms (Chapter 63).[33,145] Antibodies used in IMS may be targeted against flagella or somatic antigens. Although the sensitivity of IMS does not allow the reliable or effective capture of low numbers of target microorganisms directly from a food homogenate, it may be used to

- reduce assay time by capturing cells from a pre-enrichment broth and using these to inoculate a selective agar plate and thus avoid the selective enrichment stage[18]
- improve test performance by harvesting target cells from a pre-enrichment broth and inoculating selective enrichment broths[140]
- harvest from a selective enrichment broth for plating onto either selective differential agar media or for use with an alternative method of detection[14,17,90,171]
- aid in salmonella serotyping.[39]

Supermagnetic particles for IMS are available from commercial sources (eg, Dynabeads™, Dynal AS, Oslo, Norway). The particles are available either ready-labeled with attached homologous antibody to a foodborne pathogen, or labeled with antispecies antibody (eg, sheep anti-rabbit) for the subsequent coupling of another capture antibody by the user.[39,53,90]

The use of IMS to harvest *Escherichia coli* O157 from enrichment broths has significantly improved the isolation of this bacterium.[21] Thus, Wright et al.[177] reported a 100-fold increase in sensitivity over the standard method when using Dynabeads labeled with anti-*E. coli* O157 antibodies to detect *E. coli* O157 in minced beef. While IMS has been employed extensively for the isolation of salmonella from foods,[106,138,146,150] Hanai et al.[66] reported that only about 10% of the salmonella cells present in pre-enrichments were recovered by IMS. A high degree of nonspecific binding, particularly to *Citrobacter freundii* and *Enterobacter cloacae*, has been reported to occur on anti-salmonella Dynal™ immunomagnetic beads. Therefore the binding times and washing protocols must be optimized to minimize this effect.[14,34,177] Immunomagnetic separation also has been used to capture listeria from enrichments prior to plating[151] or combined with the Vitek Immunodiagnostic Assay System Enzyme-Linked Immunoassay (VIDAS) technique to detect *Listeria* species.[103] Table 62–8 lists some of the recent reports on the effect of introducing an IMS stage on the performance of traditional and alternative microbiological detection methods.

Table 62–8 Examples of the Improvement in the Detection of Foodborne Pathogens Using Immunomagnetic Separation Followed by Conventional Plating or Alternative Detection Methods

Pathogen	*Food Types*	*Contamination*	*Enrichment*	*Detection Method*	*IMS After*	*Positive Results*		*Reference*
Escherichia coli 0157	Beef	Spiked <10 to 100 cells 25 g^{-1}	m-EC+n	CT-SMAC	None	19/30	63%	Bennett, MacPhee & Betts[14]
			m-EC+n	CT-SMAC	m-EC+n	26/30	87%	
			m-EC+n	O/T EHEC-TEK ELISA	M-EC +N	21/30	70%	
			BPW+VCC	CT-SMAC	BPW+VCC	24/30	80%	
	Beef	Spiked 0.1 cells g^{-1}	m-BPW	MSA-BCIG	m-BPW (10h, 24h)	6/6	100%	Restaino et al.[136]
			m-BPW	Ab-DEFT	m-BPW (10h)	6/6	100%	
			m-EC+n	USDA plating	none	4/6	66%	
			m-EC+n	O/T EHEC-TEK ELISA	M-EC+n (24h)	6/6	100%	
	Beefburger	Natural	m-BPW+VCC	CT-SMAC	After 6h	3/25	12%	Bolten, Crozier, & Williamson[21]
				CT-SMAC	After 22h	1/25	4%	
			m-TSB @ 37°	CT-SMAC	After 6h	2/25	8%	
				CT-SMAC	After 22h	1/25	4%	
			m-TSB @ 42°	CT-SMAC	After 6h	3/25	12%	
				CT-SMAC	after 22h	2/25	8%	
Listeria monocytogenes	Dairy products	Natural	m-LEB–FB	MOX & PALCAM	None	0/6	0%	MacPhee, Bennett & Betts[103]
			UVM1-FB	EIA Foss ELISA	TSYEB	1/6	17%	
	Various meats, fish, vegetables	Natural	UVM1-FB	MOX & PALCAM	None	14/44	32%	MacPhee, Bennett & Betts[103]
			$^1/_2$ FB-FB	EIA Foss ELISA	TSYEB	17/44	39%	
Yersinia enterocolitica	Beef and pork, whole and minced	Spiked 10-20 g^{-1}	None	PCR-DIANA	Direct	7/7	100%	Kapperud, Vardunt, & Skjerve[90]
		Spiked 2-20 g^{-1}	TSB	PCR-DIANA	TSB	7/7	100%	
Salmonella species	Poultry, meat, liver, skin, and swabs	Natural	ISO 6579 = R-V + SC	BGA + XLD	None	31/48	64%	Cudjoe, Hagtvedt & Dainty[32]
				BGA + XLD	R-V and SC broths	45/48	94%	
				M broth—O/T-Salmonella	None	6/48	12%	
				TEK	R-V and SC broths	45/48	94%	
	Poultry–various	Natural	BPW	RVS—BGA & XLD	None	20/100	20%	Cudjoe & Krona[33]
					BPW	39/100	39%	
	Minced chicken, lamb, beef, pork	Natural	EIA Foss	Automated ELISA	M-Broth	27/96	28%	Holbrook et al. Unpublished data.
			Selective enrichment	BGA + XLD (control)	None	29/96	30%	

Key to abbreviations:

Methods:

Ab-DEFT = fluorescent antibody staining and epifluorescence microscopy; O/T EHEC = Organon Teknika enterohemorrhagic *Escherichia coli* enzyme linked immunoassay; EIA Foss = Foss automated Salmonella ELISA incorporating immunocapture; PCR-DIANA = polymerase chain reaction—Detection of immobilized amplified nucleic acids

Media:

Broths

m-EC+n = modified EC broth + novobiocin; BPW+VCC = buffered peptone water + vancomycin, cefixime, and cefsoludin; BPW = buffered peptone water; m-BPW = modified buffered peptone water; FB = Fraser broth; $^1/_2$ FB = half strength Fraser broth; m-LEB = modified listeria enrichment broth; m-TSB = modified tryptone soya broth; TSYEB = tryptone soya broth + yeast extract; TSB = tryptone soya broth; M-broth = Manitol broth; RVS = modified Rappaport-Vassiliadis soya broth; UVM I = University of Vermont primary enrichment media

Agars

CT-SMAC = sorbitol MacConkey agar + cefixime and tellurite; MSA-BCIG = MS agar + 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; MOX = Oxford agar + moxalactam & colistin sulphate; PALCAM = PALCAM listeria selective agar; BGA = brilliant green agar; XLD = xylose lysine desoxycholate agar

Semisolid Selective Media. Selective motility enrichment techniques (SMET) use the migration of targeted motile (flagellated) bacteria through or over semisolid selective media to isolate them.[156] Test systems can be set up either by using the Craigie tube principle with migration within the agar[31] or by the migration over semisolid medium in a Petri dish.[42] Semisolid selective media are formulated by adding agar or other gelling agents, such as xanthan or alginates, to the selective enrichment medium.[76] The concentration of both the selective agents and the gelling agent is optimized for motility with the possible addition of chemoattractants to enhance performance.[102] Both methods have been exploited for the detection of salmonella.

The Craigie U-tube principle originally was devised to select motile organisms from a poorly motile culture for serotyping purposes.[31] Subsequently, with the addition of antisera, it was used to select clones in the specific phase or nonspecific phase to complete immunological identification of salmonellas based on the Kauffmann-White scheme.[91] Today, the apparatus consists of a test tube or bottle having an inner tube, open at both ends, that is partly immersed in the semisolid growth medium. The device is inoculated inside the inner tube with culture together with appropriate antisera and the motile culture in the opposite phase is recovered from outside the tube.[67] The procedure also enhances antigenic expression. The Craigie tube principle has been used by a number of workers. Harvey and Price advised the use of secondary enrichment through semisolid Nutrient agar as a routine practice to improve the isolation rates of salmonella from clinical, animal, food, and environmental samples;[68,156] colonies from selective agar media were passed through the Craigie tube and plated onto further selective media. Chau and Huang developed semisolid enrichment media from both Rappaport-Vassiliadis medium and a semisolid indicator medium, based on H_2S formation, for screening food samples for the presence of salmonellae.[27] SMET was used by Holbrook et al.[76] to devise a test kit for the detection of salmonella in foods within 42 hours. The kit consisted of a culture vessel containing an elective medium in which were suspended two tubes with porous discs containing different enrichment media in their lower chambers and different indicator media in their upper chambers, both containing a salmonella chemoattractant.[102] The vessel was inoculated with pre-enrichment and after incubation positive tubes were identified by color changes; this test also allowed latex agglutination tests to be carried out directly on the bacteria in the upper chamber.[76]

The use of semisolid selective media in petri dishes has been reported for the isolation of *Campylobacter* species[65] and salmonella from fecal samples, the latter using semisolid Rappaport (SSR) and semisolid selenite medium.[64] De Smedt et al.[42] subsequently evaluated the performance of semisolid modifications to Rappaport-Vassiliadis medium in petri dishes. Evaluation was made using naturally contaminated meat, egg, and cocoa bean samples following pre-enrichment. The optimum formulation modified semisolid Rappaport-Vassiliadis medium (MSRV), containing novobiocin, gave a 39% increase in positive isolations compared with tetrathionate brilliant green enrichment, and could detect <100 salmonella ml^{-1} in the presence of 10^7 other microorganisms ml^{-1}.[43] In a comparison of MSRV with SSR media for the isolation of salmonella from coastal waters, MSRV gave significantly more positives.[129]

Swaminathan et al. first described the use of salmonella antisera-coated discs on semisolid Dulcitol brilliant green novobiocin motility medium to indicate the presence of salmonella by a line of immobilized growth around the disc.[157] Davis and Wray incorporated the antiserum disc into MSRV and recommended inoculating below the semisolid agar surface to form a liquid "bleb" to inhibit the spread of other microorganisms.[38] Many collaborative studies have now been conducted on a range of foodstuffs comparing MSRV against different standard methods.[10,19,43,173]

62.9.2 Solid Media

The gel strength of solid media is not only important for its practical use but it also affects the rate at which nutritional components diffuse to the growing colonies, thus influencing both their size and shape; it must therefore be carefully standardized for use by the manufacturer. The gelling quality of agar is dependent on the source of the natural raw material and the manufacturing process. The optimum concentration of agar for general use is between 1.2% and 2.0%. Solid media in food microbiology have three primary uses:

1. in quantitative methods for enumeration of microorganisms in food samples, using either the spread-plate or pour-plate method or other means of obtaining countable colonies such as by the spiral plate method,[56] the drop plate method (Miles and Misra technique),[29] or the Perifilm™ plate method
2. in qualitative methods by direct streaking of food or environmental samples, or enrichment

cultures or broths onto agar plates or membranes, to obtain separated colonies to find targeted species
3. to obtain pure cultures by the subculture of colonies onto plates for identification purposes.

Quantitative Methods. Counting is done by either the spread-plate or pour-plate method, each having different attributes. The spread-plate method can be used with opaque media and avoids exposing the inoculum to mild heat stress as occurs with the pour-plate method. Many differential features of media may only be well displayed by surface colonies growing under the appropriate aerobic, microaerophilic, or anaerobic conditions. Only a small inoculum, ideally 0.1 ml, can be used on a spread plate in a standard 90 mm petri dish. Where low counts are expected, and to improve detection and precision, larger sample volumes of up to 0.3 ml can be surface plated. Excessive drying of plates prior to inoculation, so that larger volumes are absorbed, is poor practice that can result in significant changes in composition of a medium; also, the risk of colony inhibition by excessive amounts of food debris present from the inoculum must be avoided. Large-size petri dishes can be used to spread up to 1 ml of a food sample.

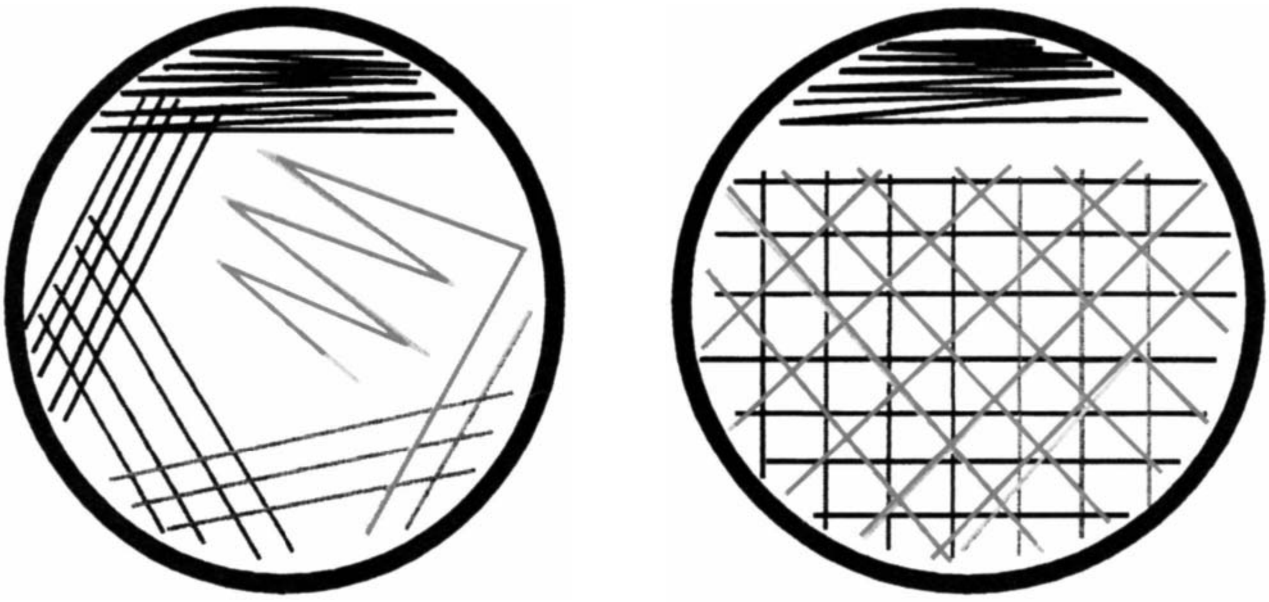
With the pour-plate method, a larger inoculum volume, typically 1 ml, is used in a standard petri dish. Cooling the medium prior to pouring is a critical step and should be monitored reliably. Exposure of the inoculum to molten agar at 45° C to 49° C followed by rapid mixing before cooling to set on a horizontal surface, is generally acceptable for determining counts for mesophilic and thermophilic species. However, for psychrotrophic species a maximum temperature of 47° C is critical for the subsequent growth of some species. It is often good practice to overlay pour plates with a thin layer of the same medium to prevent the spreading of colonies on the surface of pour plates. The inclusion of 0.0005% triphenyltetrazolium chloride to media used in a pour plate can aid the differentiation of bacterial colonies from food particles. The dye is reduced to a red formosan within each colony, giving it a reddish color and aiding observation.[158]

Enumeration may also be made by the spiral-plating method.[56] The apparatus allows an ever-decreasing volume of inoculum to be deposited in the form of an Archemides spiral over the agar medium from the center to the edge of the plate. This procedure allows a range of up to 10^4 cells ml^{-1} to be counted from a single dilution on one plate. Following incubation, colonies are counted using a template that divides the plate successively into quadrants and then into arcs of various length within each quadrant, so that areas containing sufficient numbers of separated colonies can be counted to give a statistically reliable result. The total count is computed from the regions counted.[50,56,127]

It is generally accepted that for total counts, plates containing between 30 and 300 colonies are most suitable for counting on surface and pour plates, this number being reduced to 20 to 150 colonies for the enumeration of indicator organisms and pathogens as nontargeted colonies may also be present. For statistical aspects of such count procedures, see Chapter 60; and for the assessment of test attributes such as sensitivity, linearity, critical level, limit of determination, selectivity, and specificity, see the literature.[3,28,80,81,119,162]

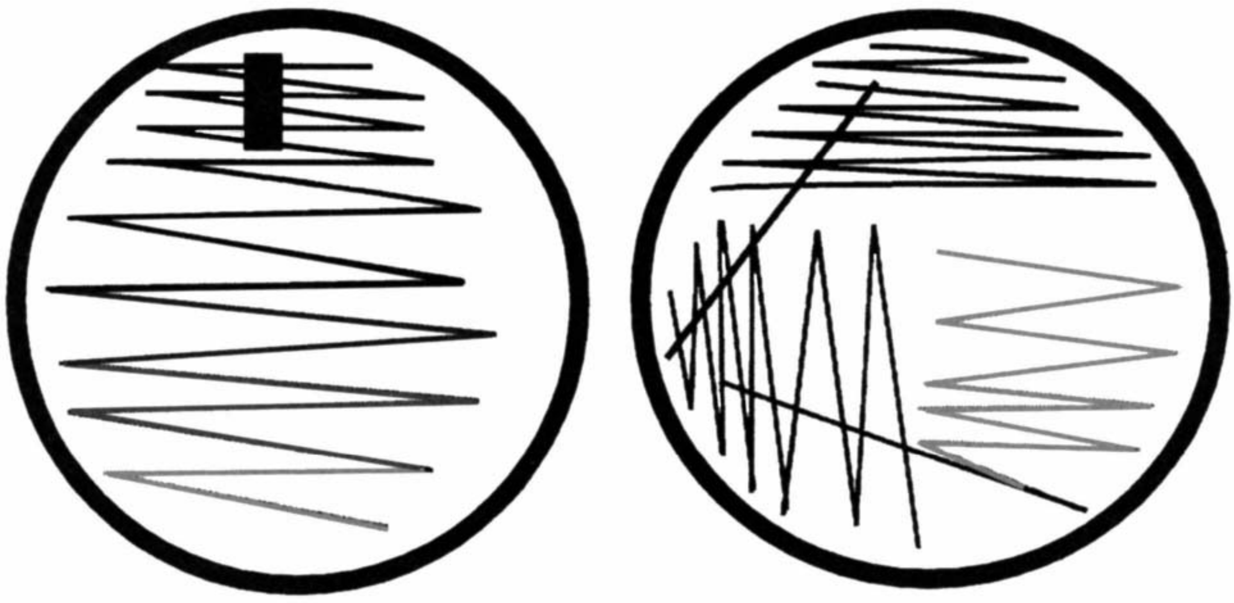
Qualitative Methods. To maximize the chance of isolating the targeted organism from an enrichment broth upon subculture to selective agar, various methods of "streaking out" have been prescribed. The principle is that ever-decreasing numbers of cells are spread over an increasing surface area of the agar to obtain discrete, well-separated colonies that show the features of identification (eg, colony shape and size, color [from pH indicators], hemolysis, proteolysis, lipolysis). The inoculum size, governed by the size of the inoculation loop, is significant and the procedure may be done with or without the sterilization of the loop between each sector. Four methods are compared in Figure 62–2. Method 1 is widely used to check the purity of a culture and comprises five, four, three, then two parallel streaks around the periphery of the petri dish away from the loop unloading area, and then a single tortuous streak over the center of the agar plate. Methods 3 and 4 are of the same principle but use different patterns to give different rates of decreasing density. Method 3 is advocated in ISO Standard 6579, 1997[86] and method 4 in ISO Standard 7218, 1996.[84] Method 2 attempts to achieve a greater distribution of colonies by using the loop to trace away from areas previously inoculated with higher numbers of cells to increase the likelihood of separating the targeted organism from these less favorable cell ratios. For example, methods 1, 2, 3, and 4 gave about 60, <100, 30, and 30 well-separated colonies respectively from the same bacterial suspension.

In all these methods there is a direct relationship between the chance of gaining isolated colonies of the target organism and the numerical ratio it has to the other organisms able to grow on the medium from the inoculum. There is, however, considerable



Method 1.
Widely used method to check a culture for purity by gaining separated colonies.

Method 2.
Repeated streaking across previously inoculated areas to gain greater separation of colonies from dense areas.



Method 3.
Inoculation of selective agars according to ISO Standard 6579, 1997.[86]

Method 4.
Inoculation of selective agars according to ISO Standard 7218, 1996.[84]

Figure 62–2 Methods of Inoculating Petri Dishes. The decrease in intensity of each line represents a decrease in inoculum.

personal opinion on the best spreading technique.

Nonselective Agar Media. These media may be formulated with[107,116] or without[24] differential properties or biochemical indicator systems but a detailed examination of the appearance of the colonies as classically described[174] gives some degree of colony differentiation and possible species recognition by an experienced user. Nonselective media used in a qualitative manner are also appropriate for the recovery and growth of injured or attenuated cells.

Selective Agar Media. A comprehensive description of the wide range of selective differential agar media relevant to food microbiology have been described.[30,96] The characteristics of a good selective differential medium include the ability to give typical colonies from a wide range of strains of the targeted organism(s), with maximum inhibition of competitors, and good differentiation from those competitor species not so readily inhibited.[41,71]

Chromogenic Media. New or modified media based on the use of chromogenic and fluorogenic substrates have been developed for the detection, differentiation, or identification of bacteria, yeasts, and molds. These may give faster detection times than conventional procedures. These synthetic substrates are colorless prior to their hydrolysis by specific enzymes. For reviews of the application of fluorogenic and chromogenic substrates in bacterial diagnosis, see the literature.[40,105] The action of the targeted microbial enzyme on a single substrate can achieve much greater specificity than indicator systems based on changes in pH. For this reason, claims have been made that confirmation tests on colonies giving specific substrate reactions in an appropriately selective medium are no longer necessary. Fluorogenic substrates containing coumarin-based derivatives of 4-methylumbelliferyl-β-D-glucuronide (MUG) or 7-amino-4-methylcoumarin (7-AMC) have been incorporated into several media, but as the free fluorochrome is soluble in water and diffuses readily, it is not ideal for use in agar media but very appropriate for liquid media using presence/absence tests or most probable number estimations. Fluorochromes that are easily demonstrated by long-wave UV (366nm) light are available in a range of compounds having different enzyme substrates. MUG is widely used to demonstrate β-D-glucuronidase activity by *E. coli* in lactose broth[58] and lauryl sulphate broth.[48] β-D-glucuronidase activity is present in about 96% of *E. coli* strains,[48,172] but is absent in verocytotoxigenic *E. coli* O157 strains.[160] β-D-glucuronidase activity also is present in some strains of other members of the Enterobacteriaceae, including salmonella (29% of strains), shigella (44%), and Flavobacteria.[59] β-D-galactosidase catalyzes the breakdown of lactose, a feature used to identify coliforms, and 4-methylumbelliferyl-β-D-galactopyranoside (MUGAL) is used as a fluorogenic substrate for this.[15,26] Some other species of the Enterobacteriaceae, including some *Citrobacter* spp., *Enterobacter agglomerans*, and *Serratia marcescens*, while not fermenting lactose, produce galactosidases.[26] Chromogenic substrates that are insoluble or precipitated following hydrolysis are ideal for use in agar media so that color is retained within, or close to, the colony. Strongly colored molecules offer better visualization and discrimination and thereby improve sensitivity. By the appropriate combination of substrates, different enzyme reactions can be demonstrated in the same agar medium, enabling the detection of two or more species or groups at the same time—for example coliforms and *E. coli.* Many new compounds are being synthesized to commercially exploit the value of such chromogenic detection systems; these include 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (BCIG).[105]

An example of a medium containing two interacting identification systems is Rambach's agar,[135] which differentiates salmonella colonies from those of other members of the Enterobacteriaceae; selectivity is achieved with sodium desoxycholate. The medium contains propylene glycol, which is used by the majority of salmonella giving a decrease in pH and causing neutral red to be precipitated within the colony giving a "fuchsia" pink color. The inclusion of the chromophore X-Gal distinguishes nonlactose fermenting coliforms from salmonella by producing colonies with a blue color; organisms that use both lactose and propylene glycol give colonies with a violet color, and colonies of those that use neither, such as strains of *Proteus* and other Enterobacteriaceae able to grow on the medium are colorless. However, some less-common but nevertheless important strains of salmonella do not use propylene glycol and thus give false negative reactions. Examples are *Salmonella typhi* and some uncommon strains of *S. paratyphi* A and B, *S. typhimurium*, *S. choleraesuis*, *S. gallinarum*, and *S. dublin.*[94]

62.10 PREPARATION, STORAGE, AND USE OF CULTURE MEDIA

The reliability of bacteriological analysis depends on the correct preparation of culture media, their storage, and use. The following is a list of important

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aspects of this process.

62.10.1 Recordkeeping

The date of purchase of dehydrated base media and their other components (eg, supplements) should be recorded together with the manufacturer's codes and batch numbers, and their expiration dates. Also record when containers are opened. The rate of use of all media should be monitored so that stock size is proportionate to its rate of use and expiration date. The date of preparation of a batch of medium, sterilization data, and pH measurement should also be recorded together with other physical QA checks, sterility checks, and microbiological assessment data. The expiration date of made-up media, prepared plates, and laboratory-prepared supplements should also be recorded.

62.10.2 Storage of Dehydrated Culture Media, Supplements, and Chemicals

The instructions in the manufacturer's handbook should be followed, including storage conditions (eg, at the recommended temperature and relative humidity and away from sunlight). Media should be used in order of batch number, ensuring that lids are tightly closed after use and should not be used after the expiration date. Powders should be examined before use for any changes in appearance indicating deterioration; any caked, lumpy, or discolored dehydrated material should be discarded. When weighing out, the time that stock powder is exposed to air should be minimized, particularly if the humidity is high.

62.10.3 Water Quality

Freshly prepared distilled or demineralized water should be used and its conductivity measured. Good-quality water should have a resistivity of at least 300,000 Ω cm.[84] Stored, distilled water may have an acid pH due to the absorption of carbon dioxide; this may affect the final pH of weakly buffered media reconstituted from dehydrated material. Water from stills containing visible algal growth or processed water that is turbid should not be used. Demineralized water can become heavily contaminated with bacteria from the ion-exchange apparatus. These bacteria may produce heat-stable substances that are inhibitory to the growth of other microorganisms so ion-exchange columns should be used in accordance with the maker's instructions, and if recommended, regularly decontaminated.

62.10.4 Media Preparation

For commercial media, the manufacturer's instructions should be followed closely. A face mask should be worn, and preferably there should be an air-extract system over the work areas to minimize the inhalation of dehydrated powders. Contact of powders with the skin should be avoided. Media should be prepared in a vessel large enough to allow good mixing and rapid heating and, in the case of media containing agar granules, time should be allowed for the agar to swell before heating to dissolve. The pH of most commercial media is adjusted by the maker so that after sterilization, the pH is correct; therefore the pH should only be adjusted prior to sterilization if instructed to do so. If the pH is wrong after sterilization, the pH of the water used should be checked. The quality of most media deteriorates during exposure to heat; therefore, this should be minimized. Excessive heating of media can cause destruction of nutrients, pH drift, precipitation, weakened gel strength, and change in color, all leading to poor bacteriological performance. Autoclaves should be properly maintained and serviced by trained engineers, and thermal locks should be fitted to prevent the door being opened until the chamber temperature is below 80° C. They should have pressure gauges and a calibrated thermocouple fitted into the exhaust line, each attached to a recorder so that the temperature and pressure cycles are recorded; records should be kept. Either thermocouples within the load or indicator tapes attached to containers or baskets should be used routinely to demonstrate that a sterilizing cycle has been completed. The use of agar preparators is recommended as these are designed to minimize the exposure of the media to heat during melting of the agar and sterilization, and thus provide media of a better and more consistent quality.

Storage of Prepared Stock Media. Prepared stock media differ in their shelf life according to either the inventor's or manufacturer's recommendations. If medium is not used on the day of preparation, and there is evidence that deterioration will not occur, it should be stored in the dark at 2° to 8° C for no longer than the recommended time. Liquid media should be dispensed in sealable containers to prevent water loss and contamination. Plates of agar media should not be dried and then stored at chill temperature, as this will cause excessive hysteresis resulting in concentration of the media components and possible inhibitory effects on bacterial growth. Also, unless such plates are poured and dried in an ultraclean environment (eg, a laminar flow cabinet), they are likely to become contaminated.

Preparation for Use. The time for reheating of agar-based products should be minimized. Agar media should be cooled to 45° C to 48° C by immersion in clean water in a disinfected water bath with the water level just above that of the agar in the bottle, before addition of supplements or the pouring of agar plates. Chill-stored supplements should be brought to room temperature before opening/rehydration and/or use and handled with care; antibiotic powders can cause hypersensitivity to the user. Soluble supplements must be completely dissolved after addition of the required solvent (eg, sterile water, alcohol) and mixed well. Insoluble supplements should be homogenized before adding to the base media. Molten agar media should be used within 3 hours of cooling. Antibiotic solutions at use concentrations should not be stored for future use.

Pouring of Agar Plates. Supplements are added to the molten cool base, mixed well without generating excessive foaming, and immediately poured. Techniques are necessary that avoid extraneous contamination, and the process should take place preferably within a laminar flow cabinet. If the medium is too hot when poured, excessive condensation results. The outside of each bottle taken from the cooling bath should be wiped before pouring the medium from it. The correct volume should be poured into each dish. Media should be allowed to solidify quickly in the horizontal position and dried for a minimal time, to remove surface moisture, thus avoiding concentration of components. Exposure to sunlight should be minimized.[170] An automated system is useful for large numbers of plates.

62.11 QUALITY ASSURANCE OF CULTURE MEDIA

62.11.1 The Manufacturer's Role

Commercially manufactured dehydrated media and supplements undergo extensive quality control by the producer. This may include the testing and selection of raw materials against chemical and/or microbiological specifications, then production against standard operating procedures, following Good Manufacturing Practices (GMP) guidelines[108] and/or in compliance with documentation prepared and accredited to ISO 9000. The end-product undergoes physical evaluation and qualitative and quantitative microbiological assessment, using appropriate test strains to ensure performance to set quality standards.[108] This process is essential because of the variability that can occur in the composition of the individual nutrient components of the medium, as discussed previously, and to provide proof of media quality for internal control and/or for certification purposes.

62.11.2 The Laboratory User

Physical Tests. These include simple examination for color, clarity, pH at 20° C to 25° C, accuracy of dispensed volumes, and, for agar media, assessment of gel strength.[35]

Microbiological Tests. While it should not be necessary for the laboratory to consider repeating the extensive testing of the manufacturer, minimal testing of a qualitative or quantitative nature should relate to the proposed use of the medium and the laboratory's need for validation of performance. A minimum range of testing might include sterility checks on all media; for commercially prepared (certified) ready-to-use poured plates, inoculation in a qualitative manner with a suitable test organism on each day of use; and for laboratory-prepared liquid media, inoculation with the final two dilutions of a simple dilution to extinction series[139] of one strain and comparison of the count obtained with a suitable control. For selective liquid media, the isolation of the targeted species from a low (<10 cells) inoculum in a mixed culture of suitable competitor organisms, with or without added sterile food is appropriate. For agar media apply tests, preferably by a quantitative method, it is necessary to demonstrate that the medium supports the growth of a small inoculum of a robust target strain and also a more sensitive target stain, and that each produces the correct reactions and/or typical colony shape and size; and where appropriate, that the agar suppresses the growth of a readily inhibited nontarget organism and gives a negative response with a less well-inhibited strain.[30] For quantitative tests on agar plates, the most efficient method of analysis is use of the spiral-plate method,[56] but other methods (eg, the Miles and Misra [drop] technique[29] or the use of the ecometric plating method[114]) by skilled operators are further options.[30]

For new stock media and supplements with new batch numbers, testing should be carried out to confirm performance, either against a previous batch or against a "gold standard," to ensure the batch has an acceptable performance. When purchasing a large amount, testing of a batch prior to confirming an order may be considered. This testing would be done using a wider selection of positive and negative strains to measure the quantitative recovery of the target microorganism and the quantitative inhibition of selected competitor organisms, together with confirmation of the expected colony reactions. It can also be appropriate to undertake further testing to demonstrate that the batch is "fit for its intended use." For example, if the medium is required to detect the presence of stressed cells from processed food, and the medium is formulated to recover such cells, then this should be experimentally demonstrated. It may also be appropriate in the case of liquid media to assess, on at least one occasion, the growth rate of a targeted organism. When establishing the performance of media prepared in the laboratory from individual components, sufficient testing using protocols that validate the significant characteristics and attributes of the medium may well be appropriate.[30]

There are currently no international standard or guidance documents concerning the QA validation procedures for media used in food microbiology; therefore, some small degree of variation in the standard of performance of media from one supplier to another is to be expected. Within Europe, the European Standardization Committee (CEN TC275 Food Analysis) is preparing a guidance document for "Quality assessment and performance testing of culture media."

62.11.3 Test Strains and Methods

How well a prepared batch of medium is assessed depends on the characteristics of the test strain(s) chosen and the methods of testing. Experience within a laboratory can identify robust and sensitive targeted and nontargeted strains appropriate for each medium used. Occasionally the "inventors" of new media identify and recommend the use of particular strains; the use of such strains improves validation of media performance.[73] Robust target test strains that grow well may not readily show small deficiencies that retard the growth of more sensitive targeted strains, and robust competitor strains that grow well may not show a deficiency in selectivity, whereas the increased growth of sensitive nontargeted strains will do so. Manufacturers use a selection of test strains for each medium to meet the quality requirements for medical, food, agricultural, and other purposes. Many of these strains are housed in national culture collections. For example, for quality assessment and accreditation needs, the UK National Collection of Type Cultures at the Central Public Health Laboratory (Colindale, London, England) have available reference collections for testing media for use with general foods, dairy, water, and food spoilage. Most of the test strains recommended by the International Committee on Food Microbiology and Hygiene[35] are available in "test strip" format from the National Collections of Industrial and Marine Bacteria (Aberdeen, Scotland). However, many of these strains are not of food origin or were not originally isolated using microbiological methods used for foods. It is, therefore, appropriate to supplement these test strains with well-characterized recent isolates that have been maintained in a stable state (eg, freeze dried, or stored at –70° C or lower) and have only been subcultured a few times, so that the opportunity for phenotypic alteration has been minimal.[152] Standardization of the methods of maintaining, preparing, and using cultures for culture media assessment have been proposed by the European Cooperation for Accreditation of Laboratories.[46]

62.12 ACCREDITATION, VALIDATION, AND PROFICIENCY TESTING

62.12.1 Accreditation

It is now recognized internationally that for a laboratory to produce consistently reliable data, it must implement an appropriate program of quality assurance measures[147,155] that is recognized by the process of accreditation of the laboratory.[5] Within the European Community, member states are progressing toward a common validation procedure,[46] which in the United Kingdom (UK) is operated by the UK Accreditation Service (UKAS) provided by the National Physical Laboratory of the Department of Trade. The European Standards, EN 45000 series for testing laboratories,[118] stipulate the steps that analytical (microbiological) laboratories must take to acquire accreditation. The UKAS accreditation procedure to these standards is outlined in guidance documents.[118] These require that, for accreditation, a laboratory must produce a quality manual and a quality policy statement. The manual must contain and describe in detail the laboratory's scope of testing or analysis and describe the quality system, organization, the roles of related management, lines of communication, and the job descriptions of relevant senior staff, together with their qualifications and training. The functions of the quality manager with respect to quality audits, quality system reviews, and audit reports also must be given. The accreditation process also concerns the use, calibration, and traceability (to national standards) of physical measurements such as time and temperature, and the maintenance and use records of all equipment that is used in the laboratory's accredited methods. Methods of analysis must be fully documented and validation data provided. The laboratory facilities, maintenance, and working environment for the staff—particularly of any specialized areas—must also be documented. UKAS requires that staff are trained and must demonstrate that they are technically competent for the work they carry out. The laboratory also is required to operate an internal quality control scheme, to monitor the performance of individual microbiologists, and to participate in external proficiency testing schemes.

Laboratories may use national or internationally prescribed methods (eg, ISO Standards), alternative methods,[45] or "in-house" methods. All, however, must have a degree of validation adequate for the purpose of use. That is measurement of repeatability, reproducibility, specificity, sensitivity, and limit of detection by interlaboratory trials—thus the precision of the method is documented. The absolute accuracy of a method is not, however, the objective.[45]

62.12.2 Validation Methods

Many well-established ISO standards for the microbiological examination of food and animal feeding stuffs currently lack precision data on interlaboratory performance but are acceptable on the grounds that the methods have stood the test of time prior to standardization. However, more recently devised methods, particularly for the detection of the emerging pathogens, do not have such status. Before an ISO Standard can become a European Committee for Standardization (CEN) standard, performance data, and particularly the reproducibility and repeatability of the method, must be known and incorporated into the text. Under the Fourth Measurement and Testing program of the EC/DG XII, a validation program, "Evaluation of Microbiological Methods for Detection and Enumeration of Microbiological Contaminants in Foods," will generate the necessary performance data on ISO and CEN methods for the detection of *Salmonella* species, *Listeria monocytogenes*, and *Escherichia coli* and enumeration methods for *Bacillus cereus*, *Clostridium perfringens*, and *Listeria monocytogenes*.

Alternative microbiological methods (ie, manufactured kits) based on more recent technologies claim various advantages over traditional methods, such as speed of analysis, less expertise required, less hands-on-time, and automatability. For acceptance, these also require validation status. International organizations such as Associattion of Official Analytical Chemists (AOAC) International,[4,111] the International Dairy Federation, the Nordic Standards Organization (NMKL), and national schemes such as the French Standards body (AFNOR)[98] have in place suitable validation protocols and operational mechanisms for assessing such kits. The AOAC has a proven track record for both traditional and alternative methods[4] and is used by the US Food and Drug Administration (FDA) for this purpose. Under present development is a European-wide validation certification scheme—MicroVal. This will operate to a European CEN Standard—"Microbiology of food and animal feeding stuffs. Protocol for the validation of alternative methods," which is in preparation, uses validation data and statistical analyses produced by MicroVal pretrials.[45,137]

62.12.3 Proficiency Testing

The objective of external proficiency testing schemes is to measure a laboratory's performance of analysis using blind samples prepared by an external organization. The results are confidential but indicate the ratings of each participating laboratory. Samples relevant to the work of the laboratory are sent at predetermined times for analysis and the reported data analyzed by the organizers. In the UK, such schemes are run by the Public Health Laboratory Service (Central Public Health Laboratory, Colindale, London). The Food External Quality Assurance Scheme (EQAS) provides freeze-dried materials for five different purposes. These are for the Standard Scheme, detecting foodborne pathogens in samples; an Extended Standard Scheme of qualitative and quantitative methods where the operator decides from a given scenario which tests to perform; and three further schemes specific for shellfish, dairy, and water analysis. The UK Ministry of Agriculture Fisheries and Foods, through its Central Science Laboratories (York, England), operates a "Food Examination Performance Assessment Scheme" (FEPAS), providing verified blind samples of various dried meats containing target and competitive flora; again the results are confidential and provide independent proof of the quality of analysis achieved. Reference materials,[12] made at the Netherlands National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands), comprise gelatin capsules of dried milk powder containing high or low levels of relevant foodborne pathogens (eg, *Salmonella* species, *Listeria monocytogenes*, *E. coli* O157). These are used by addition to any foods with their own natural microbial flora. The gelatin capsules, being stable for several weeks and having naturally stressed cells from the preparation procedure, have been used in several European trials on the interlaboratory validation of methods.[10,88] Sources of reference materials for proficiency testing are available in a number of further countries. The International Dairy Federation has a standard for the assessment of analyst performance doing colony counts.[81]

62.13 CONCLUDING REMARKS

The microbiological analysis of foods is becoming far more formalized and thus less flexible, particularly in Europe, as implied in the latter parts of this text and elsewhere. While standardization of methods, accreditation of laboratories, and proficiency testing leads, hopefully, to our goal of improved reliability and a more uniform quality of analysis, will it also retard or even stifle future advances in methodology? It is inevitable that adoption of a new method will be slow. It will also be increasingly expensive to validate its performance and this will be a significant barrier to the wide adoption of a new method.

Traditional methods are criticized for being labor-intensive, slow, and requiring a reasonable level of expertise and/or experience for their proper performance and interpretation. Yet in the past 30 years, no alternative analytical method has completely, or even in a significant part, replaced them. Is the needle in the haystack (or the one pathogenic *E. coli* cell in a raw meat sample) just too elusive for methods other than those which rely on some form of growth stage prior to detection?[77] ELISA, although increasingly used as a means of speeding up microbiological analyses, has not eliminated the need for this growth stage. Will gene probes be more successful and will more sensitive direct detection methods, which may not be confirmable by traditional microbiological methods, be accepted as evidence of the presence of an undesirable microorganism, recognizing the increasing evidence that our existing media fail to detect severely injured and stressed microorganisms?[153] In the meantime, the traditionalists progress by reformulation of existing microbiological media, with an occasional new idea that makes a significant breakthrough. Such developments will continue with the dual aim of making our methods more robust and sensitive.

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63 Detection of Microorganisms in Food—Principles and Application of Immunological Techniques

David M. Radcliffe and Roy Holbrook

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63.1 INTRODUCTION

Immunological techniques historically have been used by microbiologists and pathologists for the direct and indirect detection of microbial pathogens. The high sensitivity and very high specificity of these methods can give very accurate and rapid results. In food microbiology, the main application is in the detection of microorganisms, their structural components, or toxins. Pathogenic microorganisms may occur in foods in numbers that are far below the sensitivity of any immunoassay technology. For this reason pre-enrichment and/or enrichment culture (see Chapter 62) usually is necessary prior to the use of immunological tests. This chapter describes the use of immunological techniques for the detection of microorganisms and their toxins in foods and in cultures.

63.2 MICROBIAL ANTIGENS

Antigens from bacteria may be divided into three main types according to their location: (1) those present on the cell surface and readily accessible to antibody reactions such as the O (somatic), H (flagella), K (capsule), fimbriae, and outer membrane proteins; (2) those hidden within the cell wall or deeper and inaccessible in intact organisms to antibody reactions; and (3) those exported by the bacterial cell such as bacteriocins and exotoxins.

63.2.1 O Antigens

O antigens are present in the cell wall of bacteria and are lipopolysaccharide (LPS) in nature. They are heat-stable, resistant to alcohol, and resistant to acid but destroyed by periodate treatment. Phenol or formaldehyde treatment may inhibit O agglutination. O antigens can undergo smooth to rough transformation. This is a gradual process during repeated subculture and may cause a granular pattern of growth in broth culture and changes in serology. The reaction between smooth organisms and specific O-antiserum results in a granular type of agglutination. Autoagglutinating strains may be alcohol- and heat-treated before O serotyping. LPS, found typically in the Enterobacteriaceae, are complex molecules composed of three distinct moieties, a lipid component, a core oligosaccharide, and a side chain that confers the O serotype specificity. The core oligosaccharide in most of the Enterobacteriaceae comprises heptose and 2-keto-3-deoxyoctonate (KDO).

63.2.2 H Antigens

These are flagella proteins. They are heat-labile, acid-labile, and are detached and eventually inactivated by alcohol. They are stabilized and remain immunogenic and antigenic after treatment with formaldehyde. Loss of flagellae in a culture renders the organisms nonmotile and nonagglutinable in specific H antisera. The flagella antigen reaction with antiserum produces a looser, fluffier agglutination than that produced by O antigens. Phase variation occurs in salmonellae, designating Salmonella H antigenic types into either phase 1 or phase 2. Complete serotyping may involve phase-changing techniques involving the use of phase-specific antisera. The major protein component of flagella is flagellin. For example, the flagellin protein of the H:7 antigen often associated with *Escherichia coli* serogroup O157 has a molecular weight of approximately 66kD.[96] H antigens are usually highly immunogenic and H-sera is typically high-titered and of high affinity.

63.2.3 K Antigens

K antigens are capsular polysaccharides. They can be removed from the organism by heat treatment, although they are not considered particularly heat labile. Isolates may be serotyped with K-specific antisera, but this tends to be a procedure for a specialized laboratory. K antigens can inhibit the somatic O-antigen reaction and therefore it may be necessary to use heat treatment to allow O serotyping. *Salmonella typhi* possesses the Vi capsular antigen, which is heat-stable but easily removed from the cell surface by heat treatment, thus allowing O serotyping. Agglutination of unheated organisms by Vi-specific antiserum is a presumptive identification of *S. typhi.*

63.2.4 Fimbrial Antigens

Fimbriae (pili) are bacterial appendages similar to flagella but smaller and shorter. Nonfimbriate to fimbriate and fimbriate to nonfimbriate conversions may occur in cultures; this is often related to subculturing practices. In food and veterinary microbiology, detection of the fimbrial type SEF 14 of *Salmonella enteritidis* and *Salmonella dublin* is now possible using a commercially available latex agglutination kit.[111]

63.2.5 Outer Membrane Proteins (OMP)

OMP are a heterogeneous group of proteins found within the cell wall and may be closely associated with the LPS components. Some OMP are porins and some are formed in response to starvation. All surface-located OMP are potential targets for antibody reactions.

63.2.6 Toxins

Toxins may be intracellular or extracellular and have potent biological activity. They vary in their heat- and chemical-stability and therefore some toxins may survive the cooking process. Detection of a number of toxins in food and culture has been achieved through animal feeding studies, cell culture, and immunoassay. Immunoassay may be performed on the food sample or on cultures of organisms isolated from the food. Specific treatments such as polymyxin B and lincomycin can be used to increase the production or release of toxin.[13,15]

Mycotoxins are produced by fungi via the polyketide route as secondary metabolites. A large number of mold species produce these toxins with many designated as carcinogenic. Highly potent mycotoxins produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus* are designated aflatoxin. Mycotoxin levels in food may be measured using enzyme-linked immunosorbent assay (ELISA) tests or separation and concentration for subsequent analysis may be effected using immunoaffinity columns; see also Chapter 53.

63.2.7 Fungi

The major antigens found in fungi are extracellular polysaccharides (EPS). Large quantities of EPS are produced during fungal growth in food and culture and these may be detected easily by immunoassay. The EPS of *Aspergillus* spp. and *Penicillium* spp. in common with many other fungal species consist of galactomannan. The immunodominant epitope of galactomannan is the β(1,5)-linked D-galactofuranosyl side chain.

63.2.8 Viruses

Foodborne outbreaks of viral gastroenteritis are becoming more common. The types of virus implicated include hepatitis A, adenovirus, rotavirus, and small, round, structured viruses (SRSV), including the Norwalk virus and astrovirus. Viruses contain a single nucleic acid type, either DNA or RNA. The protein or glycoprotein coat (capsid) is composed of capsomeres, which are individual protein units. Viruses also may possess a lipid membrane derived from host tissues in which viral proteins may be found in the form of spikes (peplomers). Antisera for immunodiagnostic use may be prepared against the exposed proteins and glycoproteins. Antigenic changes occur frequently in viruses, which may affect their detection. Both antigenic drift (slight change) and antigenic shift (major change) are known to occur. Viral contamination of food is generally at a level lower than detectable by immunoassay. Success has been achieved, however, in detection of virus in shellfish and aquatic bivalve mollusks that as filter feeders may concentrate virus many hundreds of times.[26] In most investigations, culture of the sample in susceptible cell lines followed immunoassay or some other test was required (see Chapter 52).

The viral capsid proteins provide the main target for specific antibody reactions. Although some direct immunoassays exist for the detection of viruses in clinical specimens, the sensitivity of such tests is too low for use in detecting foodborne viruses. Antibody may, however, be used to capture virus particles for subsequent analysis by non-immunoassay methods (see Section 63.6).

63.2.9 Protozoa

Parasitic protozoa such as *Toxoplasma, Cyclospora, Giardia, Sarcocystis,* and *Cryptosporidium* have been implicated in outbreaks of foodborne illnesses (see Chapter 51). They have complicated surface antigenicity. Due to low numbers encountered in food, the analysis for parasites generally is limited to microscopical techniques. Fluorescent antibody techniques may have a role and are currently used in detection of some protozoa in environmental samples.[88]

63.3 ANTIBODY PRODUCTION

63.3.1 General Aspects

The production of antibodies is a result of challenge to the animal of a foreign substance or substances. Natural antibody responses are a result of microbial infection or sometimes a localized chemical challenge such as certain food components. A molecular species that is used to induce an immune response in an animal is known as an immunogen. A molecule must be recognized as nonself to induce an immune response and must be presented in a suitable form and at a suitable location. An immunogen must be of a certain minimum molecular weight (approximately 5000) to induce a response. However, the actual areas on the molecule that are recognized by the immune system are relatively small and may consist of as few as three amino acids in the case of protein antigens. These regions are known as epitopes; a large and complex protein may have a number of different as well as related epitopes. The majority of epitopes are discontinuous (ie, consist of amino acids that are contiguous only in the folded tertiary or quaternary structure). The precise regions on the antibody molecules that bind to the antigen epitope are known as the paratopes and these are located at the Fab end.

Five different immunoglobulin isotypes have been identified in mammals: IgG, IgM, IgA, IgD, and IgE. Of these, only IgG and IgM have found an important role in immunodiagnostic tests for the detection of microbial antigens. The primary response to the introduction of an immunogen, either naturally by infection or artificially, may result in a limited number of antibody-producing clones of lymphocytes. As the infection proceeds, the number of clones increases, leading to a pool of circulating antibodies with a diverse range of reactivity. Each of the clones will, however, produce antibody with very specific reactivity toward a single epitope. The response of an animal to a microorganism thus may consist of the production of antibodies against a wide variety of epitopes, and the response will vary between animals. IgM is the predominant antibody in early infection. Later, or in subsequent challenges with the same organism, IgG predominates.

63.3.2 Polyclonal and Monoclonal Antibodies

The antibody produced by a single clone of lymphocytes will be consistent in affinity and avidity, always reacting with a single epitope. In 1975, Kohler and Milstein[53] succeeded in immortalizing single clones of antibody-producing mouse cells by fusing them with mouse lymphoma cells and growing them in sufficient volume where usable quantities of antibody could be prepared. This type of antibody derived from hybridoma cells is known as monoclonal antibody. The technique of producing monoclonal antibodies is highly advanced and there are a variety of methods to develop the clones and for the large-scale culture and purification of the antibody. Diagnostic tests that had previously relied on polyclonal antibodies could now use the superior specificity of monoclonal antibodies that also offer the advantages of worldwide assay standardization. Despite the increasing use of monoclonal antibodies in diagnostic test kits, there are still many, particularly in food microbiology, who use polyclonal antibodies. This is due, in part, to the high affinity and avidity of polyclonals and their clonal diversity.[46] These properties can be advantageous for microorganisms that exhibit antigenic diversity because some isolates may have reduced reactivity against a single epitope-reactive antibody. Usually, high-affinity antibodies yield better sensitivities than low-affinity antibodies.[69]

63.3.3 Antibody Form and Function

Immunoglobulin G (IgG) is a glycoprotein of approximately 150kD in molecular weight and is the most abundant immunoglobulin in mammalian serum, amounting to between about 12 and 20 mg ml^{-1}. The IgG molecule (Figure 63–1) consists of two identical heterodimers, each composed of a heavy and light chain. The heterodimers are linked by a disulfide bridge known as the hinge region. Each heavy chain consists of one variable and three constant domains. The light chains are composed of one variable and one constant domain. The variable regions that give the antibody specificity are located at the top of the Y-shaped structure; this end is known as the fragment antigen binding (Fab) region.

At the other end of the IgG molecule is the fragment crystallizable (Fc) region. This is recognized by host defense mechanisms including binding to cells of the immune system and to C1q, the first component of the complement system. It also is bound by the surface proteins of certain microorganisms, most notably Protein A from *Staphylococcus aureus* and Protein G from Lancefield group G streptococci.

The structure of IgM is similar but it is a pentamer having 10 antigen-combining sites and a molecular weight of 950kD. As with IgG, the IgM subunits are linked by disulfide bonds but also have the addition of a protein J (joining) chain. Some IgM may be in hexameric form lacking the J chain. Certain oligosaccharides are associated with IgM.

The specific binding of antibody does not cause direct harm to microorganisms but does allow host effector mechanisms to operate, of which the most important is the activation of the complement enzyme cascade system. This results in direct toxicity of complement components on bacteria as well as a localized inflammatory response leading to many defensive effects, including migration of phagocytic neutrophils and monocytes, release of opsonins for phagocyte stimulation, mast cell degranulation causing the release of histamine, and the release of

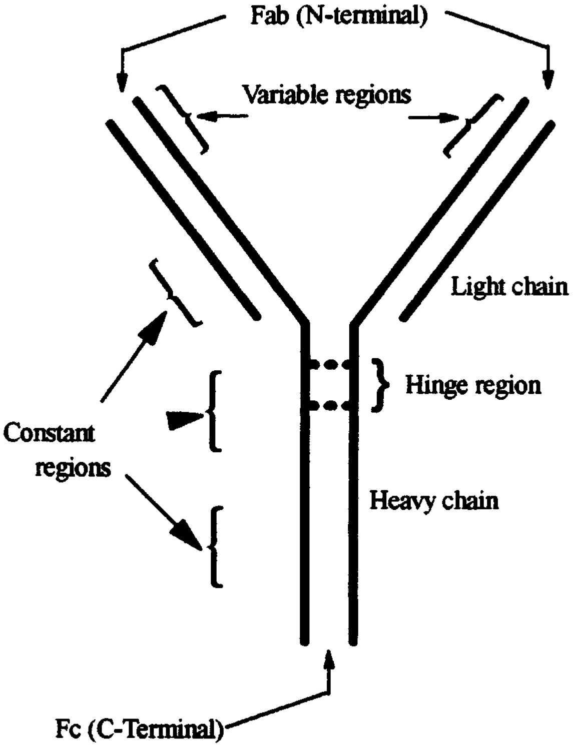


Figure 63–1 IgG Molecule. The IgG molecule has two light chains and two heavy chains attached at the hinge region. Antigen binding occurs at the N-terminal end, Fab (fragment antigen binding), which is in the variable region. The Fc (fragment crystallizable) region occurs at the C-terminal end in the constant region.

bacterial proteases. Other specific effects of antibody on bacteria may include immobilization due to the cross linking of flagella.

Antibody binds to antigen by a number of noncovalent forces at the molecular level. These forces are hydrogen bonding, electrostatic, Van der Waals, and hydrophobic. For these forces to effect binding, the epitope and paratope must be in very close proximity; this therefore requires both to have a complementary shape. From this, it follows that shapes and chemical groupings that are less complementary result in lower affinity attachment. The number and type of molecules that form the epitope also are important in defining the strength of attraction. Epitopes showing minor variations will bind to greater or lesser extents. Homogenous epitopes and complementary paratopes will give maximum binding for that configuration.

63.4 THE APPLICATION OF IMMUNOLOGICAL TECHNIQUES IN FOOD MICROBIOLOGY

In the detection of microbial antigens using antibodies, the key feature used in all tests is the specific binding of antigen and antibody. The differences between the wide variety of available assays are the methods of detection of that binding. No immunoassay formats have been specifically designed for food microbiology, but a number of methods have been adapted for this use. Each immunoassay has its own requirements, advantages, and disadvantages according to its application—whether for food microbiology or otherwise. Table 63–1 illustrates the range of sensitivity of several immunological methods used to detect the enterotoxins of *Staphylococcus aureus*, *Clostridium perfringens*, and the botulinum toxin of *Clostridium botulinum*.

Many of the other available immunoassay technologies could probably be extended for use in food microbiology.

63.4.1 Classification of Immunological Techniques

Immunological techniques include immunoassays for detection and assay of biological molecules; they include the use of antibodies as tools for capture and for the concentration of microorganisms prior to detection or culture. They also have a significant role in research for characterization of organisms and antigens. Immunological techniques may be divided into several groups. Those described here are the ones most used in immunoassays and include other methods that are not strictly immunoassays, but that use antibodies and are useful in food microbiology. A more comprehensive listing and classification has been compiled by Price and Newman.[79]

63.4.2 Immunoassay Methods

The term immunoassay is used here in its broadest sense and includes those tests used for qualitative results (ie, yes/no) as well as those used quantitatively. Quantitative immunoassays determine the amount of antigen detected in a sample, which can be used, for example, to determine the level of mi-

Table 63–1 Comparisons of the Limits of Detection of Various Immunological Methods for the Demonstration of Bacterial Food Poisoning Toxins

Immunological Method	*Staphylococcal Enterotoxins A & B µg ml⁻¹*	*Botulinum Toxin Type A Mouse 50% Lethal Doses ml⁻¹*	Clostridium perfringens *Type A Toxin µg ml⁻¹*
Precipitin techniques			
Double diffusion: Microslide	0.1	370	0.3
Tube method	0.6	—	0.013
Electro-immunodiffusion	0.5–0.15	140	0.01
Counter current immunoelecrophoresis	1.0	100	0.002
Agglutination techniques			
Reverse passive hemagglutination	0.001	27	0.00005
Latex agglutination	0.1	—	—
Radioimmunoassay	0.001	5–10	0.001
Enzyme-linked immunoassay	0.001	10	0.000001

crobial toxin(s) in a food sample. Most of the available assays give qualitative results by giving either a simple positive or negative signal or by determining whether the result is above or below a specified value. Divisions in immunoassay type may be made between reagent-limited (generally competitive) and reagent-excess (immunometric). A division also may be made between homogenous and heterogenous assays. In homogeneous immunoassays, the test sample antigen and reagent antibody are mixed to bring the antigen and antibody together; there is no separation of free from bound reagents. The result is then obtained directly. These immunoassay techniques include some of the oldest and most simple tests, such as agglutination and precipitation. The limited number of steps, short reaction times, and relatively low cost make them popular methods in many microbiology applications. Heterogeneous immunoassays account for most of the immunology-based diagnostics used today. After reaction of the test sample and reagents, one or more steps are used to separate the free unreacted label from the bound label. The requirement for separation steps generally leads to longer assay times than in homogenous tests but the separation of free label from bound label gives better potential for high sensitivity and the removal of the food matrix during the process can improve, and is often essential for, specificity. By separating each of the reaction and detection stages, the development of such tests is simplified, with common reagents being used for a number of related assays. ELISA is the most common example of heterogenous immunoassays.

Heterogeneous immunoassays may be divided further into immunometric (reagent-excess) and competitive (reagent-limited) types. In an immunometric assay, there is a capture step, generally employing a specific antibody. Antigen present in the test sample binds onto this capture antibody and a labeled antibody is added. The detection of binding of this labeled antibody determines the result of the reaction. An increasing level of signal is indicative of an increasing quantity of analyte in a direct relationship. Competitive immunoassays use a labeled (tracer) antigen that competes with the sample antigen for binding to a (generally) solid-phase antibody. In a competitive assay, the signal level is reduced by increased concentrations of the antigen in the sample because this sample antigen allows less tracer antigen to bind to the solid-phase antibody. Alternatively, a solid-phase immobilized antigen is used to capture labeled antibody in the presence of sample antigen. If antigen is present in the sample in sufficient quantity, it binds to the labeled antibody, thus stopping it binding to the solid phase. Labeled antibody is therefore removed in the washes, thus reducing the signal strength after substrate addition.

Antibodies, like many other proteins, may be attached easily to solid surfaces and thus provide an effective means for capturing homologous antigen molecules. Washing the surface then removes unbound material, including excess antigen, other antigens, and irrelevant or interfering matrix material. If it is multivalent (more than one antibody binding site), the bound antigen can react with further antibodies. The second antibody, when linked with an indicator molecule to form a conjugate, will show whether it bound and therefore whether the target antigen was present originally. In this immunometric, heterogenous type immunoassay, an excess amount of the anti-analyte reactants is desirable. A large excess of solid-phase antibody can bind a large amount of target antigen. A large excess of the second tracer antibody will then be more likely to saturate the bound analyte. With a fully saturated system, the signal generated will be at a maximum and, within the designed working range, will correlate with the quantity of bound antigen.

The labels used to prepare the indicator antibodies include radioactive isotopes, enzymes, fluorophores, chemiluminescent molecules, and small particles. Enzyme labels commonly used are listed in Table 63–2. The substrates that produce a soluble reaction product are used in visual and instrument-read assays. The precipitating substrates produce insoluble colored reaction products and may be used for membrane-based diagnostics including immunochromatography, dot-blots, and for visually read ELISA tests.

63.4.3 Detection of Reactions

The majority of homogeneous assays, especially of the agglutination type, are read visually or by nephelometry. Heterogenous assays frequently, but not exclusively, use instruments for reading of results. Modern immunochromatography tests are examples of immunometric, heterogeneous, visually read tests. Enzyme immunoassay (EIA) reactions produce a colored result and, although it is sometimes possible to read by eye, greater low-end precision is obtained by a spectrophotometric measurement. Both fluorescence immunoassays (FIA) and chemiluminescent immunoassays (CLIA) produce a light output at specific wavelengths with FIA requiring excitation light. Both of these methods require instrumentation that therefore enables quantitative mea-

Table 63–2 Enzyme Labels and Substrate Systems Commonly Used

Enzyme Label	*Substrate*	*Reaction Product*	*Use*
Horseradish peroxidase	O-phenylenediamine (OPD)	Soluble	ELISA
	2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)	Soluble	ELISA
	3,3',5,5'-Tetramethylbenzidine (TMB)	Soluble	ELISA
	3,3'-Diaminobenzidine tetrahydrochloride (DAB)	Insoluble	Immunoblotting
	3-Amino-9-ethylcarbazole (AEC)	Insoluble	Immunoblotting
	4-Chloro-1-napthol (4C1N)	Insoluble	Immunoblotting
	3-(*p*-hydroxyphenyl)propionic acid (HPPA)	Soluble	Immunofluorescence
Alkaline phosphatase	*p*-Nitrophenyl phosphate (*p*NPP)	Soluble	ELISA
	5-Bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT)	Insoluble	Immunoblotting
	Fast red/naphthol AS-TR	Insoluble	Immunoblotting
	4-Methylumbelliferyl phosphate (4-MUP)	Soluble	Immunofluorescence
β-Galactosidase	*o*-Nitrophenylgalactoside	Soluble	ELISA
	4-Methylumbelliferyl galactopyranoside	Soluble	Immunofluorescence
Urease	Urea + bromophenol purple or phenol red pH indicator	Soluble	ELISA

surement. In addition, some commercial immunoassay systems are fully automated and include bar code specimen ID, automated reagent additions, measurement of result, and printout or data output to a computer. For foodborne microbial antigens, the VIDAS system (bioMerieux), Opus (Tecra), and EIAFoss (Foss Electric) are examples of automated immunoassays.

63.5 EXAMPLES OF IMMUNOASSAYS

63.5.1 Agglutination

Agglutination reactions require solid particles, in this case the whole target bacteria. Colonies are mixed into saline on a glass slide to produce a smooth suspension. Specific antiserum is added (usually hyperimmune rabbit or goat serum), and the slide is rotated gently to provide very slow mixing, allowing antigen and antibody to join. Because IgG molecules are divalent and IgM molecules are pentavalent, an antibody may attach to two or more antigens (organisms), allowing the rapid development of large, lattice-type structures (Figure 63–2) that are easily visible to the naked eye. Some isolates are prone to autoagglutination; thus a control test is necessary, in which the organism is mixed into saline or normal rabbit serum.

Greater levels of sensitivity can be achieved in a tube agglutination where the reaction between antibody and antigen is performed in a larger volume of liquid. The tubes are incubated, generally at 37° C, partially immersed (one third) in a water bath to give gentle mixing by convection currents. Incubation times range from 1 to 24 hours; results generally are read by eye but can be measured using a spectrophotometer (reduction in absorbance due to agglutination) or a nephelometer (reduction in light scattering due to agglutination). Alternatively, a microwell plate method may be used,[99] particularly for testing of isolates against a large number of antisera. This provides good control over the technique and economic use of reagents. The results may be read by eye (with optional magnification) or in a microwell plate spectrophotometer. Agglutination tests are used extensively for the identification and serotyping of a wide range of foodborne pathogens. Examples of the use of agglutination test systems for pathogenic bacteria are listed in Table 63–3.

63.5.2 Passive Particle Agglutination

Principle. When specific antibody is coupled to small particles, for example latex particles, it retains antigen-binding activity and the visibility of aggluti-

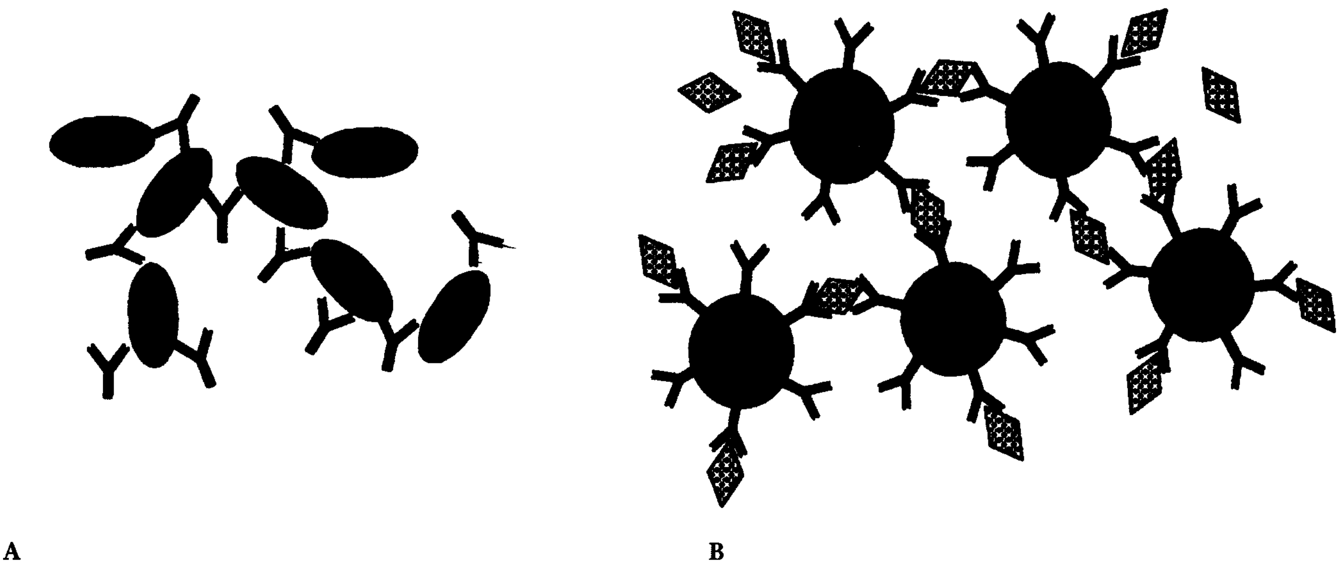


Figure 63–2 Agglutination Reactions. **A**, Agglutination of bacterial cells. Cross-linking by specific divalent IgG molecules causes the formation of clumps. **B**, Passive particle agglutination. Specific IgG on the surface of latex particles is cross-linked by soluble antigen molecules.

nation is enhanced and sensitivity increased. Passive particle tests can be used to detect both soluble and particulate antigens. The principle of passive particle agglutination is illustrated in Figure 63–2. Many types of particle may be used. The chief requirements are:

1. small, uniform, particle size (<1000 nm diam.)
2. suitable color or refractive index to be visible against a contrasting background
3. capable of being coated with protein (sensitization)
4. remaining as discrete particles after sensitization
5. the antibody after sensitization must retain antigen reactive capabilities.

Commonly employed particle types include red blood cells (RBC), latex particles, Staphylococcal Protein A, and colloidal gold.

Red Blood Cells. RBC membranes are particularly suitable after chemical treatment to receive protein sensitization. These cells are treated to fix the cell membrane, making them resistant to lysis and more stable, plus surface modification to accept antibody sensitization. Treatments may be with trypsin or neuraminidase, fixation with formaldehyde, glutaraldehyde, or dimethyl suberimidate, and membrane surface modification with tannic acid.[56] After treatment, antibody is attached to the cells by incubation and any uncoated sites are blocked with a suitable protein solution such as bovine serum albumin or casein. Blocking is necessary to avoid nonspecific binding. RBC agglutination tests have been developed for many organisms, including *Campylobacter*,[76] *Clostridium perfringens*,[36] and *Aeromonas* spp.[40]

Latex Particles. These particles, produced by the emulsion polymerization of organic monomers, have the advantage of high degrees of monodispersity. They are available with a wide range of surface properties, sizes, and colors. For simple passive sensitization, the latex suspension is diluted in a suitable buffer and mixed with antibody solution. Coupling of the antibody is rapid and occurs within 5 to 30 minutes, although final reagent stability may not be achieved for another 24 hours. Washing the particles and blocking of unsensitized sites usually are necessary. Polystyrene particles are most commonly used for passive sensitization. Where difficulties are encountered in attachment of antibody, a covalent method of sensitization may be employed.[48,68,80] Carboxylate-modified particles have surface-bound carboxyl (COOH) groups that may be chemically coupled to amino groups on antibody molecules using water-soluble carbodiimides. Other covalent meth-

Table 63–3 Agglutination Test Systems for Typing Some Foodborne Pathogens

	Antigens		*Agglutination Method*			
Foodborne Pathogen	*Type*	*Number*	*Slide*	*Tube*	*Tray*	*Commercially Available*
Bacillus cereus	H	>19		✓	✓	No
Clostridium perfringens	K	>72		✓	✓	No
Campylobacter jejuni/coli/lari	Heat-labile	108	✓			Limited range
Aeromonas species	O	97		✓	✓	No
Escherichia coli	O	>176	✓	✓	✓	Limited range
	K	>10	✓	✓	✓	
	H	>50	✓	✓	✓	
Listeria monocytogenes	O	15	✓	✓		Limited range
	H	5	✓	✓		
Salmonella species	O	>66	✓	✓	✓	All the common groups
	H	>44	✓	✓	✓	
	Vi	1	✓	✓	✓	
Shigella boydii	O	15	✓			Limited
Shigella dysenteriae	O	10	✓			Limited
Shigella flexneri	O	11	✓			Limited
Shigella sonnei	O	1	✓			Yes
Yersinia enterocolitica	O	>30	✓			?
Vibrio cholerae	O	>83	✓			Limited
Vibrio parahaemolyticus	O	11	✓			Limited range
	K	>51	✓			
	H	?1	✓			

ods are available but less frequently used. The very high affinity of biotin for avidin (or streptavidin) may be useful in preparation of latex reagents,[95] because this avoids harsh treatments that can damage antibody. Colored latex particles can provide enhanced visibility due to increased contrast.

A considerable number of latex agglutination tests, both commercial and in-house, have been developed for use in identifying organisms, including *S. aureus*,[32] *Campylobacter jejuni* and *C. coli*,[43] *E. coli* serogroup O157,[61] *E. coli* heat-labile enterotoxin,[34] *C. perfringens* type A enterotoxin,[63] *Vibrio cholerae* O1,[108] *V. parahaemolyticus*,[14] and *Salmonella* spp.[41] Latex agglutination has been used successfully to detect *Trichinella spiralis* antigen in pork samples[16] and for detection of *Penicillium* and *Aspergillus*.[51]

Staphylococcal Protein A (SPA). Protein A is an antibody-binding protein found on the surface of *S. aureus* cells; analogous proteins are found on some other species, notably Protein G on some Lancefield group G Streptoccocci. *Staphylococcus aureus* cells grown to high density, and when fixed, provide a suitable solid phase for the attachment via the Fc end of IgG. Coagglutination is the usual term for agglutination tests employing SPA. Relatively few immunological tests have used SPA but they have the advantage of correct orientation of IgG molecules after coupling. A coagglutination test for pathogenic *Vibrio* species was developed that used four species-specific monoclonal antibodies for the identification of *V. mimicus*, *V. vulnificus*, and *V. cholerae*.[101] Both the heat-stable and heat-labile antigens of *C. jejuni and C. coli* have been used for serotyping by coagglutination[117] and a commercial kit is available for Lancefield grouping of streptococci.

Passive particle agglutination tests usually are read by eye using glass or disposable card or plastic slides. Whole cells are the usual target but antigen extracts may be used. The sensitivity of such tests rarely exceeds 10^7 colony forming units (cfu) per ml, but this is not usually a problem when testing colonies or cultures and the tests are simple, rapid, and relatively inexpensive. Better sensitivity has been achieved in the detection of soluble antigen.[51]

Passive Particle Tube Agglutination Tests. Tube tests have been developed using RBC (hemagglutination), gold sol, and latex particles. These assays use the differences in the settling pattern of antibody-coated particles when they are exposed to antigen. Salomon and Tew in 1968[91] developed a test for the detection of *Staphylococcus* enterotoxin B in which the sample is mixed with antibody-sensitized latex particles and incubated overnight. Agglutination is detected by observation of the sediment pattern. This type of agglutination test uses microtiter plates and antibody-coated latex particles for the detection of microbial toxins either in food or from liquid broth cultures of food isolates. These reversed passive latex agglutination (RPLA) tests have the advantage of relatively high sensitivity but with visual reading of the results. When agglutination occurs, the large clumps settle to the base of the well but friction resistance stops the clumps from rolling down to the base of the V-shaped well. Where no agglutination occurs, the discrete particles, as they settle out, roll down to the bottom of the V-well. Measurement of the amount of toxin is achieved by adding the latex suspension to a series of dilutions of the sample. The highest dilution giving a positive result is used to calculate the toxin level in the sample. RPLA tests have been developed for Staphylococcal enterotoxins,[87,98] *C. perfringens* enterotoxin,[1] *V. cholerae* and *E. coli* heat-labile enterotoxin,[92,105] *Bacillus cereus* diarrheal enterotoxin,[4,5,11,25] and *E. coli* verocytotoxins 1 and 2. The incubation time for RPLA tests is 20 to 24 hours; however, a microtiter plate centrifugation technique can cut the time to just 4 hours.[3]

63.5.3 Precipitation Reactions

Liquid Phase Reactions. Where a reaction develops between soluble antigen possessing multiple antigenic determinants and specific soluble antibody, cross linking and lattice formation occur. The ratio of antigen to antibody molecules is much more critical than in agglutination and an excess of either may result in a failure to develop sufficiently large complexes to become insoluble and therefore visible. Tube precipitation tests are, therefore, uncommon and performing precipitation tests within a gel matrix is more usual.

To prevent antigen or antibody saturation, a series of dilutions should be made of both antigen and antibody solutions in 0.01M, pH 7.4, phosphate buffered saline. Each dilution of antiserum is mixed with each dilution of antigen to form a checkerboard pattern. Incubation is at 37° C for 24 hours or longer (the minimum detectable levels improve with further incubation). The dilution steps should be close together (eg, neat, 1/2, 1/4, 1/8) to avoid missing critical equivalence points. Precipitation may be improved by the addition of 3% polyethylene glycol (PEG) of MW 6000. The identification of Lancefield group D antigens in enterococci by tube precipitation has now largely been superseded by latex agglutination.

Immunodiffusion Reactions. Diffusion within a gel allows gradations of the concentration of either or both of the ligands. As the antigen and antibody diffuse toward each other, areas of optimum antigen/antibody ratio develop and visible precipitation occurs at appropriate concentrations. Agarose gels are most frequently used as the matrix. Agarose is a purified derivative of agar, and aqueous agarose gels are economical, easily prepared, and allow the free movement of antigens and antibodies, including the larger molecules such as IgM. Agarose gels also are nontoxic, therefore allowing excised precipitin bands to be used for animal inoculation. Agarose usually is prepared at 1% (w/v) for general immunodiffusion work. Lower concentrations can lead to weaker gels that are difficult to work with, and higher concentrations can inhibit molecular mobility and increase the reaction time. The composition of the liquid phase used to prepare the gel is not too critical; however, salts are required to maintain ionic strength and the pH should be within normal values for antigen/antibody reactions. Therefore, suitable solutions include phosphate buffered saline (PBS) and barbitone buffer. The addition of 3% PEG will improve precipitin bands. With increasing temperature, diffusion speed increases but the resolution of precipitin bands diminishes. Most immunodiffusion assays are therefore conducted at 4° C; this also reduces the opportunity for microbial contamination to affect results. A preservative such as 0.1%

sodium azide may be added. Two immunodiffusion techniques are in common use:

- **Gel Double Diffusion (GDD).** This is useful for detecting specific reactions between antibody and antigen and for showing whether apparently identical reactions are related. The usual pattern is a central well surrounded by six equidistant wells. This central well contains either an antiserum or an antigen preparation with the peripheral wells containing the opposite analyte. The antigen/antibody ratio is very important, and several dilutions of both antisera and antigens should be prepared. Once the antiserum dilution has been optimized, future work using this dilution may require only minimal variants of antigen concentration. The line patterns show whether the antiserum in adjacent wells shares complete, partial, or no homology (Figure 63–3). When lines cross, there is no shared identity; when there is a complete joining with a rounded corner, there is complete identity and the detected epitope is reactive with the same antibody in both wells. If the same complete identity pattern occurs but with an additional spur, this is interpreted to indicate an antigen-reactive antibody in addition to that found in the adjacent well.
- **Radial immunodiffusion (RID).** In RID, the antiserum (about 0.5%) is added to the agarose while still molten but cool enough to avoid antibody damage (ideally below 50° C). When set, wells are cut in the gel and the antigen preparations added. The antigen diffuses into the gel containing constant antibody concentration. As it diffuses, precipitation and therefore immobilization occur. Unreacted antigen continues to diffuse until it complexes. The size of the precipitin ring is therefore dependent on the concentration of antigen. RID is often used to measure the amount of antigen present in a sample by constructing a standard curve using known concentrations of standard antigen. It is also possible to add antigen to the agarose with serum in the well for the measurement of antibody concentration. This is known as reverse RID (RRID) and may be used, for example, in comparison of batches of antiserum.

Perhaps the major use in food microbiology for agarose diffusion methods has been the identification of Staphylococcal enterotoxins (SET). A culture filtrate of the *S. aureus* isolate is placed in the central well and antiserum reactive with the major SET types A, B, C, D, E, and possibly others are placed in the peripheral wells. With subtype-specific antisera, further identification is possible. Modifications, such as the microslide gel double diffusion test,[12] and techniques to improve the analysis of larger numbers of isolates have been introduced.[64,104]

63.5.4 Immunofluorescence

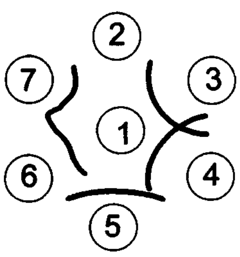
The use of antibodies labeled with fluorescent dye was introduced by Coons in 1941.[18] The fluorescent antibody (FA) technique has been applied to the rapid detection of salmonellae in centrifuged samples of enrichment broth and also by a modified method of plating the enrichment culture onto brilliant green agar for detection by microcolony FA.[110] An indirect immunofluorescence assay was developed to detect five of the major fungal spoilage organisms in tomato purees.[84] The assay reduced errors associated with the Howard mold counting technique. A fluorescent sandwich immunoassay using a portable fiber optic sensor was developed to detect staphylococcal enterotoxin B in ham and other samples. This allowed onsite analysis of suspect food samples.[109] Fluorescent methods are highly sensitive, but their use is limited due to the natural fluorescence of many materials found in samples and the environment. Techniques to improve the signal-to-noise ratio include the use of fluorophores with a high Stokes shift (the difference between the excitation and emission wavelengths) and time-resolved fluorescence using lanthanide chelate labels such as europium where, as well as the long Stokes shift effect, the fluorescence is longer lived allowing for natural fluorescence to be filtered out by the instrumentation.[35]

63.5.5 Membrane-based Immunodiagnostics

Defined porosity membranes in materials such as nitrocellulose, polycarbonate, polysulphone, and nylon are used commonly in filtration and more recently in immunodiagnostic tests. Protein such as antibody may be bound to the surface of the membrane allowing use in colony-blot and dot-blot tests using enzyme immunoassay reagents. Colony-blot methods have been developed, for example, for heat-labile enterotoxin-producing *E. coli* isolates[94] and *C. botulinum* toxin.[39]

Flow-through devices[114] generally have a capture antibody bound in a defined region to a membrane. The sample is added and allowed to flow through the membrane into absorbent pads on the other side. A

Key:
Well 1 = Antiserum
Wells 2–7 = Antigens



Reactions:
2 No reaction; 3,4 Reaction = No identity;
4,5 Reaction = Partial identity between the two antigens;
6,7 = Complete identity, likely to be the same antigen.

Figure 63–3 Gel Double Diffusion

specific antibody-enzyme conjugate is added followed by substrate. A colored result indicates a positive result. Variations of the technique include use of antibody-bound latex particles to provide an increased surface area for antigen capture. These tests are generally qualitative, but by use of a reflectance meter a quantitative result may be obtained.

Lateral-flow devices use the ability of some membrane materials to permit liquid to flow along their length. The term immunochromatography has been applied to assays using this process. Enzyme conjugates or colored latex particles can be used as the indicator of binding. The original latex, lateral flow device[22] consists of a specific antibody (usually monoclonal) bound to the membrane in a thin line at a region along the strip. At the sample application zone, there are colored latex particles sensitized with another specific antibody. These particles are free to move in the capillary flow of liquid within the membrane. When sample liquid is added, the antigen, if present, will bind to the latex particles and will move along the membrane strip in the liquid flow. The antigen will bind, together with its colored latex label, to the immobilized antibody line, giving a defined colored line. A second antibody line further along the strip consists of an antimouse IgG antibody that will bind the monoclonal sensitized latex particles directly and thus will act as a control. This test has been applied to the detection of *Listeria monocytogenes* in enrichment culture.[83] Such lateral flow tests have just two steps, the addition of a sample and reading the result a few minutes later.

63.5.6 Enzyme-Linked Immunosorbent Assay

ELISA tests, also known as EIA tests, use enzymes conjugated chemically to antibody as the tracer. They are generally heterogeneous immunoassays although there are some homogenous assays, the best-known of these being enzyme multiplied immunoassay technique (EMIT). In clinical pathology, some assays are used to detect specific antibodies produced in response to infection. To detect microorganisms or toxins in food or culture, both immunometric and, less frequently, competitive tests are used.

ELISA tests all follow certain basic principles. A typical assay consists of a number of specific binding steps and a number of wash steps to remove unbound material (Figure 63–4).

The most common format for ELISA tests uses 96 microwell plates. Also available are strips of 8 or 12 wells that snap into plastic holders and are useful for conducting fewer tests. A wide variety of instrumentation is available for the microtiter plate format including multiple well pipettors, plate washers, and multiple wavelength spectrophotometric plate readers. Programmable robotic systems can be purchased also to automate much of the work. Macrobeads and microbeads may be used as the solid phase, and a variety of dipstick type formats are also available.

It is necessary to go through a lengthy optimization process to develop ELISA tests, including choice of the solid phase, selection of the capture and conjugate antibody, selection of the conjugate enzyme and substrate system, conjugation method, incubation times and temperatures, washing steps, and reading the result of the reaction.

Enzyme-linked immunoassays offer the opportunity to reduce the assay time required for the detection of many foodborne pathogens that produce a specific antigen suitable for this test. Also, where technical experience is lacking for colony recognition of pathogens on selective agars, its replacement with an ELISA may improve reliability. Numerous reports since 1977[54] on the performance of laboratory prepared and commercially manufactured ELISA methods are available in the literature. Enzyme-linked immunoassays have been described for most of the major foodborne pathogens. Because of their sensitivity, enzyme-linked immunoassays are ideal for the detection of bacterial toxins. Examples are the demonstration of *B. cereus* diarrheal toxin,[25] *C. perfringens* enterotoxin A,[113] the heat-labile enterotoxin,[89] and heat-stable enterotoxin[86] of *E. coli* and *S. aureus* enterotoxins.[70]

Many commercially produced ELISA kits are now available for the detection of foodborne pathogens and toxins. While the performance of many of these kits has been compared with recommended or standard methods, comparisons between kits for detect-

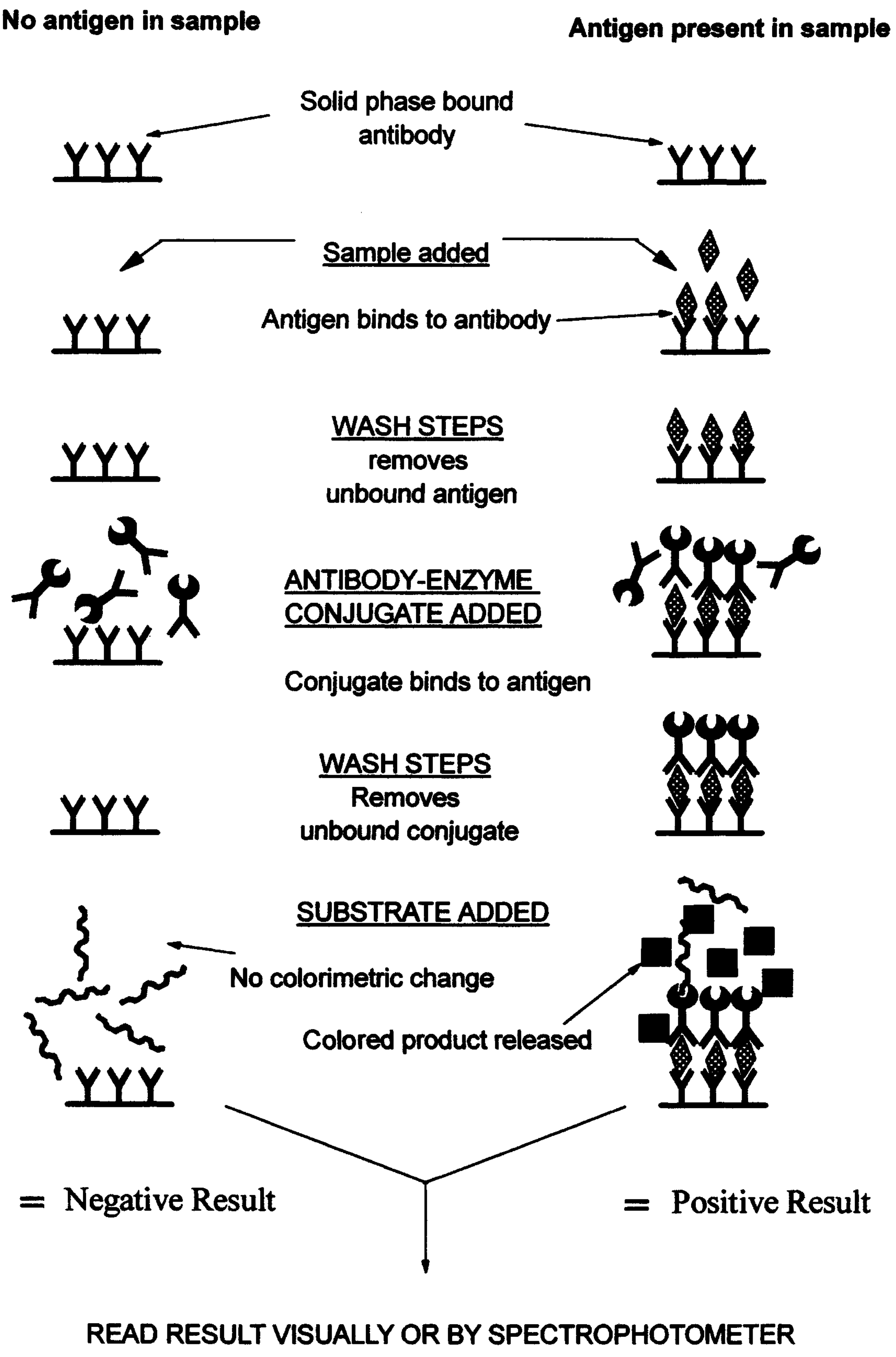

No antigen in sample
Antigen present in sample
Solid phase bound antibody
Sample added
Antigen binds to antibody
WASH STEPS
removes unbound antigen
ANTIBODY-ENZYME CONJUGATE ADDED
Conjugate binds to antigen
WASH STEPS
Removes unbound conjugate
SUBSTRATE ADDED
No colorimetric change
Colored product released
= Negative Result
= Positive Result
READ RESULT VISUALLY OR BY SPECTROPHOTOMETER

Figure 63–4 General ELISA Schematic

ing the same microorganism or toxin are limited.[31] The minimum number of bacteria that can be detected in a standard sandwich ELISA is generally $\sim 10^5$ cfu ml^{-1}. Culture in selective enrichment broth (with pre-enrichment where advocated) usually is necessary to give such numbers, but the selective conditions may result in poor expression of the target epitope. Subculture into another, often nonselective, medium is then necessary to stimulate antigen expression and reduce any interference from food. If the target antigen is hidden or particulate (eg, a cell-wall component or flagellum), treatment to render it in a reactive (soluble) form is necessary. Such treatment often multiplies the number of target molecules significantly by depolymerization giving increased test sensitivity. Automation of the assay[55,72] and methods to reduce the period of culture are being sought continuously to achieve a total test time of <24 hours.

In the analysis of foods or a culture supernatant, for the presence of a toxin several factors contribute to the quality and thus the assurance of the assay. Amplification of ELISA tests can improve their sensitivity. In the most well-known example, an alkaline phosphatase antibody conjugate is used for the dephosphorylation of nicotinamide adenine dinucleotide phosphate (NADP) to nicotinamide adenine dinucleotide (NAD). Alcohol dehydrogenase reduces the NAD to NADH and diaphorase converts it back to NAD with the simultaneous reduction of p-iodonitrotetrazolium violet to a purple formazan dye. The continuous cycling of the NAD reaction causes the formation of hundreds of formazan molecules. A similar amplification technique was used to increase the sensitivity of an assay for the *Clostridium botulinum* neurotoxin from food,[100] allowing it to be used as a replacement of the mouse bioassay. Another amplification technique, also for *C. botulinum* neurotoxin, used ELISA-ELCA, an ELISA-enzyme-linked-coagulation-assay.[28,29,85]

63.5.7 Enrichment Serology and Motility Enrichment

A number of researchers have developed methods for the rapid detection of salmonellae using a combination of pre-enrichment, enrichment, and immunological stages.[9,106] The common factors in most of the methods are the selective growth of presumptive salmonellae in a highly motile form (motility enrichment) and the detection by *Salmonella* polyvalent H sera or reagents prepared from such antisera. Various serological tests have been used including standard ELISAs, fluorescent antibody, filter-membrane based ELISA,[54] and latex agglutination.[45] A collaborative study on the use of modified semisolid Rappaport-Vassiliadis (MSRV) medium and serological detection of *Salmonella* from foods was performed by De Zutter et al.[27] Several commercial products are now available that are based on the enrichment serology principle.[21,44] The enrichment serology technique has been extended to include other foodborne pathogens of major concern, such as *Listeria* by ELISA[62] and *Campylobacter* by latex agglutination.[81]

63.6 THE USE OF IMMUNOMAGNETIC SEPARATION (IMS) TECHNIQUES IN FOOD MICROBIOLOGY

Superparamagnetic iron oxide Fe_3O_4 particles can move within a magnetic field (yet lose their magnetism when the field is removed); thus when a magnet is placed at the side of a tube containing a liquid medium, suspended particles will rapidly move to that area. Antibody is attached to either ferric oxide particles or ferric oxide incorporated within a polymeric shell (paramagnetic latex particles) to provide an antigen capture reagent. The use of such particles is a very effective means of separation and concentration. Washing steps, to remove food material and contaminating antigens, are also very easily performed. Antigen may be eluted from the particles, usually by reduced pH treatment. The concentrated and purified eluted antigen can be detected by a conventional immunoassay or alternatively the antigen-coated particles may be used in an immunoassay without elution. Stannard et al.[107] developed a magnetic enzyme immunoassay (MEIA) for Staphylococcal enterotoxin B. This used polyacrylamide agarose beads containing entrapped iron oxide particles. Enterotoxin captured from processed food samples on the particles was detected by an antibody-coupled peroxidase.

Antibody-sensitized paramagnetic particles have been proposed for the capture of live bacteria from food samples.[37] To a sample of food, specific antibody-(usually antisomatic or antiflagella antibody) coated paramagnetic particles are added. An incubation step allows binding of the target organisms to the particles. After separation and wash steps, the now concentrated and cleaned suspension is pipetted onto agar culture media, streaked-out, and allowed to grow normally. Alternatively, samples are pre-enriched in buffered peptone water prior to IMS of target organisms; this is followed by selective enrichment before plating onto selective media. Or the particle-bound organisms are placed into pre-enrichment, then enrichment, culture prior to solid media culture. The separation of target organisms from most of the rest of the sample results in a reduction of competing microflora. This therefore can aid recovery of the target organisms particularly when they are sublethally injured. IMS has been used for capture of live salmonellae,[19,60,82,102,115] *V. parahaemolyticus*,[112] *Listeria* spp.,[2,30,65,103] *S. aureus*,[49] and *E. coli* O157.[6]

IMS can be combined with other technologies such as the polymerase chain reaction (PCR) for *Yersinia enterocolitica* O3,[52] Salmonellae,[116] *E. coli* O157,[47] and *Coxiella burnetii* in cows.[67] A technique has been developed for the detection of hepatitis A virus (HAV) in American oyster, in which human anti-HAV antibody coupled to magnetic beads is used to capture HAV from extracts for subsequent analysis by PCR.[58] Yu and Bruno[119] used an immunomagnetic-electrochemiluminescent system for the detection of *E. coli* O157 and *Salmonella typhimurium* in food samples with a stated sensitivity rate of 10^3 CFU ml^{-1}. Immunomagnetic separation has been used to aid the flagella phase inversion that is necessary for the H-serotyping of salmonellae.[23] The detection of type A enterotoxin of *C. perfringens* in food extracts has also been achieved using IMS.[20]

IMS is a powerful technique that has been shown in most studies to radically increase both the sensitivity and specificity of organism detection and can reduce analysis time. For the detection of *Listeria* from environmental samples Mitchell et al[65] found that the use of immunomagnetic beads detected *Listeria* in 100% of samples at contamination levels where a standard cultural method gave detection in only 36% of samples. Also, quantitative results were produced within 24 hours for both positive and negative samples as no enrichment was required, while for the standard cultural method qualitative results required 6 days for positives and 3 days for negatives. In contrast, Poppe et al.[78] found only a little improvement in isolation of *Salmonella* from raw poultry samples. When optimized, the IMS technique yielded 65% positives compared with 60% by conventional methods. Mansfield and Forsythe[59] found in a comparison of an IMS method and a conventional enrichment method for the detection of salmonellae in herbs and spices that of 468 samples analyzed, 195 (41.7%) were positive by both methods. Eighteen were positive only by IMS enrichment and 19 only by the conventional method and not IMS. As with other immunodiagnostic tests, IMS requires high-specificity antisera for the efficient separation of target bacteria from background contamination. In a study[17] to compare conventional culture with IMS methods for the detection of *Salmonella* in sausages it was found that although IMS improved the detection by almost two-fold, there was a high level of false positives due to *Citrobacter freundii*, some serotypes of which share common antigens with *Salmonella*.

A high rate of nonspecific binding (NSB) by immunomagnetic beads has been observed also in some studies. The use of wash steps employing specific additives has been found useful to eliminate NSB. Tween 20 (0.002% to 0.005%) has been used to aid the IMS of *E. coli* O157 from beef mince[118] and Tween 20 (0.1%) plus 4% skim milk for *Salmonella livingstone*.[115] The skim milk was found to be effective at blocking NSB due to *Aeromonas hydrophila*. Reviews of magnetic separation methods have been given by Olsvik et al.[73] and specifically for food by Safarik et al.[90]

63.7 STANDARDS

The use of controls or standards is very important in immunoassays. Their use shows that each reaction step and each washing step of the assay has been conducted correctly. The minimum requirements for controls are a known high-level calibrator antigen, a known low-level calibrator antigen, and the sample diluent (= negative control). Additional controls could include further known antigen levels to provide a standard curve, appropriate food sample to exclude false positive results, and real or artificially inoculated food or culture preparations.

63.8 FACTORS AFFECTING IMMUNOLOGICAL TECHNIQUES

Strain Variation. Due to the heterogenous nature of microorganisms, variations in antigen production will occur between strains. In plate cultures of *E. coli* capsular serotype K1, two types of colony were found with different reactivity toward K1 antiserum. One form ($K1^+$) gave strong agglutination, the other ($K1^-$) gave weak agglutination; the $K1^+$ form contains the polyneuraminic capsular polysaccharide in an O-acetylated form.[74] Genotypically motile organisms including salmonella may fail to produce flagella in culture, and motility enrichment is a useful technique to increase the numbers of actively motile bacteria. Some isolates of salmonellas are, however, incapable of producing flagella. The O111 inag-

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glutination phenomenon is found in live cultures of *E. coli* serogroup O111 where such large quantities of free O-polysaccharide are produced that the antibody is saturated, thus eliminating the agglutination of the whole cells.[38]

Temperature. Immunological reactions, in common with other chemical reactions, are affected by temperature. With increasing temperature, the rate of antigen/antibody collisions increases, leading to increased capture or binding. An opposing force also will occur, because due to the protein nature of antibody, increasing temperature will lead to increasing thermal denaturation. This also occurs with heat-labile antigens. Small, thermally induced changes in the structure of antibody or antigen can cause partial or complete loss of binding and hence reduction in the sensitivity and speed of reaction. Thermal effects also will affect other proteinaceous materials used in immunoassays such as enzyme conjugates; again, increasing temperature causes increasing rate of catalysis but also increases thermal denaturation. Nonprotein materials also may be affected, in particular the substrates used in ELISA tests. Conversion of substrate to colored product is greatly accelerated by contact with enzyme but will take place slowly in the absence of enzyme. An alteration in temperature from that specified by the manufacturer or that validated in a trial will shift the assay sensitivity and specificity and may cause an increase in false positives or false negatives. Many tests are conducted at room temperature for ease of use but this is an ill-defined value and could range from below 10° C to above 30° C depending on location, time, and laboratory facilities. Wherever possible, the immunoassay laboratory should be maintained between 18° C and 22° C. The ambient laboratory temperature at the time the assay is conducted should be logged to aid in tracking discrepant results.

The storage temperature of immunoassay reagents is very important. For the majority of commercial kits using liquid reagents, refrigeration is required. The expiration date on the product is derived from shelf life studies (stability tests). Usually for each 10° C rise in temperature, the effective shelf life of the kit will halve. For some reagents, freezing will preserve their activity beyond the stated expiration date; for others including particle-based assays, especially latex suspensions, freezing, even momentarily, may cause complete and irreversible autoagglutination.

Repeated freeze-thawing of reagents is particularly damaging; a better policy is to freeze in small aliquots, which may be removed from the freezer and used once only. When preparing standards for future use in checking product sensitivity, it should be borne in mind that even freezing will not maintain the effective strength indefinitely. The best way to maintain nonparticulate standards is by freeze drying, to achieve a low moisture level, and then by subsequent storage in a frozen state. When immunoassay reagents are removed from cold storage, it is advisable to bring them to room temperature before use. If this is not done, the rate of reaction will be slower, causing reduced sensitivity and possibly false negative results. This is of particular importance for rapid tests that may reach completion time before attaining the correct temperature.

Growth temperature of microorganisms can affect expression of antigens; for example, the putative flagellin from *L. monocytogenes* is expressed abundantly when grown at 20° C, but only in trace amounts at 37° C.[75]

pH. In general, the optimum pH for antigen-antibody binding steps in an immunoassay is at physiological pH, 7.3 to 7.4; however, antibody binding activity is surprisingly tolerant of pH variations. Denaturation of antibody and antigen will occur at much higher and lower pH values. In a population of polyclonal antibodies, this denaturation pH may be spread over a wider range; for monoclonals a tighter cut-off is to be expected. Some food samples may be too acidic for efficient detection by immunoassay of microorganisms or their toxins. Best results are obtained by neutralizing the sample before testing. Extraction or dilution in buffer should effectively neutralize acidic food samples.

Time. Almost all immunoassays rely on timed steps. Deviation from these times will cause changes in the sensitivity/specificity balance. With ELISA tests, if the incubation times are reduced, lower binding will occur. Latex agglutination tests always have a cut-off time, usually 1, 2, or 3 minutes. The sensitivity is set by the manufacturers to provide at the limit time a division between positive and negative results. If the slide is not rocked for sufficient time, weak positives may be missed. Results after the limit time should be ignored as nonspecificity will increase with time. Although immunochromatographic tests have a built-in control line, the tests require further incubation up to the manufacturer's limit time to detect weak positives because even after the control line has formed, there is a continued flow of liquid along the device.

Extraction Efficiency. Microorganisms in food may be hidden effectively from detection due to their being deeply embedded within the matrix. A key factor in detection of microorganisms and their products in food is an efficient means of separating the target from the matrix. Methods of food extraction can be found in Chapter 61. The availability of antigens may be increased by specific heat and/or chemical treatments. For example, heating *S. typhimurium* at 100° C in 50mM ethylenediaminetetraacetate (EDTA) was found to dissociate its antigens into a form that resisted sedimentation at normal centrifuge speeds and caused a marked increase in the rate of immunoreaction and sensitivity in an enzyme immunoassay.[8]

Ionic Strength. The antigen-antibody reaction involves weak, physical, noncovalent bonds, and the primary and specific reactions involve coulombic and Lifshitz van der Waals forces. The coulombic bonds in particular are sensitive to both changes in ionic strength and pH.[24] Optimum ionic strength lies generally around that of physiological solutions.

Viscosity. The reaction between antigen and antibody only occurs when the reactants are in very close proximity. The rate of reaction is dependent on the number of specific bindings. With a high viscosity medium, convection currents and Brownian motion will be reduced, and the number of reactions will be reduced, causing a lower rate of reaction and therefore lower sensitivity. Agglutination reactions in particular are severely affected because they depend on the coming together of relatively large particles to form larger agglomerates. The movement of these particles through a viscous medium is impeded and, as agglutinate size increases, the movement reduces further.

Cross Reactions. Cross reactions are caused by unwanted but true antigen/antibody reactions that cause false positive results. There are two basic types of cross reaction.

- **Type 1**. The specific antibody reacts not only with the target organism but with an identical epitope present on another organism or present in the food substance or culture medium. Attempts to remove this cross reaction by absorbing the antiserum with the nonspecific organism will result in reduction or elimination of both the nonspecific and the specific reaction. Other polyclonal serum batches or modifications in the vaccine preparation may produce better specificity. It is therefore the aim of most monoclonal development programs to prepare antibodies with reactivity toward unique epitopes.
- **Type 2**. A polyclonal antiserum having antibody that reacts with the specific target epitope may also possess antibody reactive with minor antigens on the target organism. These minor antigens may be present on other organisms (as minor or major antigens) or as elements of the food or culture medium and, therefore, the antibody will react with them. Absorption of the serum with the cross-reactive organism usually will result in improved specificity without loss of the specific antibody. There is a known cross reaction between Staphylococcal enterotoxins A and E.[7] There are cross reactions between *E. coli* O157 antisera and the *Salmonella* O30 serotype.[97] Some strains of *Escherichia hermannii* are found to cross react with *E. coli* O157 antiserum.[10] This is related to the common occurrence of an N-acyl derivative of 4-amino-4,6-dideoxy-D-mannopyranosyl residues in the O-polysaccharide fractions of the LPS of the respective organisms.[77]

Nonspecific Binding. Nonspecific binding is caused by one component of an assay binding to another, but the term excludes a specific reaction between antigen and antibody. In latex agglutination tests, blocking unused sites on the latex particles after sensitization of antibody is necessary. If unblocked sites remain, protein may bind nonspecifically and cause cross-linking, resulting in false-positive agglutination. In ELISA tests, the NSB of the antibody-enzyme conjugate to the solid phase would cause a false positive signal. Even with perfectly blocked solid phases, a degree of NSB may occur and cause the assay to be less specific than desired. This is a severely limiting factor in homogenous assays as interfering substances, if present, are not removed from the assay. In heterogenous assays, the washing steps can remove most potential interfering substances. The inclusion of a detergent, usually Tween 20, can increase the effectiveness of washing and thus aid specificity. The improvement in specificity will also allow for tests to give better sensitivity.

Washes. If there are wash steps in an assay, the manner in which they are conducted can affect the assay sensitivity, specificity, and precision. For some assays, particularly those detecting large macromolecules, overvigorous washing can detach antigen and result in reduced sensitivity. Conversely, inadequate washing can result in increased NSB and therefore reduced specificity.[93]

Dilution. The amount of sample dilution will affect the assay sensitivity. Dilution may be required to mitigate the effects of sample interference. Concentration of the sample may be used to increase sensitivity.

Chemical Interference. Samples that contain substances such as lipids, detergents, or acids may reduce the sensitivity or specificity of assays. Sample purification may be required to reduce the effect of such substances. Lysozyme causes nonspecific results by cross linking IgG molecules.[33] This lysozyme interference may be overcome by the use of Cu^{2+} ions and ovalbumin.[71]

Protein A and Protein A Like Substances. Protein A present in the cell walls of *S. aureus* will bind the Fc end of IgG,[66] and its presence as a contaminant in a sample may cause interference in an immunoassay. Nonspecific agglutination of antibody-coated latex particles is a notable example. The use of Fab fragments as an alternative to whole IgG will remove this problem. Some Lancefield group G streptococci possess a similar Fc-binding substance known as protein G. The cyclic adenosine monophosphate (CAMP) factor of *Streptococcus agalactiae* binds to IgG and IgM.[50]

Hook Effect. An excess of antigen in a sample can cause a reduction in reactivity in some assays, particularly those based on particle agglutination and precipitation. This is known as the hook effect, named after the shape of the dose-response curve; it is also called the prozone phenomenon. Antigen excess causes saturation of the antigen-binding sites on the antibody, thus limiting cross-linking of particles and reducing the signal. For those assays that may be subject to this effect, either the sample will need to be prediluted or a number of dilutions should be prepared and tested.

Cross-Well Contamination. The microplate format having wells within very close proximity can suffer from cross-well contamination. A small volume of concentrated sample could cause false positives in adjacent wells. It is therefore important to ensure that the sample is added without causing splashes or aerosol formation. Urease conjugates have been used occasionally in enzyme immunoassays with a urea substrate and pH indicator for signal detection. Possible false positives may be caused due to ammonia liberation affecting the pH of adjacent wells.[57]

Product Drift-Stability. Reputable diagnostic kit manufacturers go to great lengths to establish the effective shelf life of their products, including studies simulating transportation conditions. It is therefore important that products are only used within their stated expiration date. It is likely that in-house produced assays are not so rigorously tested for stability and their sensitivity and specificity are likely to change with time.

Antigen Adherence. Antigens may adsorb onto the surface of glass and plastic containers and on filter membranes. If a sample contains a very low level of antigen, this adsorption can significantly affect the result and may cause false negatives. It has been found beneficial to preblock containers with solutions such as 1% bovine serum albumin.

ELISA Plate Readers. Microplate readers, often assumed to offer precision and accuracy, have been found to produce erroneous results, particularly in well-to-well bias.[42] Therefore it is important to determine the precision, accuracy, and well-location bias in a microplate reader. Regular servicing and calibration are required.

Food As a Sample. Food is a highly diverse material and the complex mixtures of proteins, carbohydrates, fats, preservatives, and other additives in foods may cause a number of effects on immunodiagnostic tests. When testing the sensitivity of an assay on a new food type, it is necessary to inoculate that food with varying known levels of organism or toxin and measure the minimum detection level. This also extends to the use of immunodiagnostic tests on enrichment culture where the foodstuff is still present.

Culture Media As a Sample. Culture media are much less complex than foods. Detection of microorganisms in foods usually involves pre-enrichment followed by enrichment and then isolation on solid media. The indirect detection of food-poisoning organisms using immunodiagnostic tests traditionally has been performed on isolates from agar media; however, many assays have been designed for detection of the organisms in the enrichment medium. Toxin tests may be performed on filtrates of broth cultures or organisms isolated on solid media. Culture media recommended by manufacturers of a kit should be adhered to because cross reactions, although rare, can occur. Also, in the wrong medium, insufficient growth, antigen yield, or toxin production may occur resulting in false-negative results.

Selective media, in particular, can sufficiently alter the organism's growth pattern to produce unreliable results. In such cases, subculture to nonselective media is recommended.

On solid culture media testing, a number of isolated colonies to avoid selection of an unrepresentative type is advisable. The identity of an isolate giving a positive immunoassay result should be confirmed by other means (eg, Gram stain, biochemical reactions) to reduce the possibility of a false-positive reading.

63.9 CONCLUDING REMARKS

In this chapter, the authors have given a brief review of immunological techniques and their application to food microbiology. A limited number of examples have been given that have hopefully demonstrated the advantages of speed, sensitivity, and specificity of immunoassays but also have shown the possible pitfalls and limitations of these tests. The applicability of immunological techniques to each stage of microbiological food analysis (eg, direct detection, culture detection, culture identification) is likely to increase over the coming years. Also, the use of the antigen-antibody reaction for capture of organisms from food before detection by other means such as culture and PCR shows a very promising future. As the requirement for efficient and rapid food analysis increases, validated immunological techniques will be likely to continue to form an important element of routine microbiological analyses.

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64 Principles and Applications of Genetic Techniques for Detection, Identification, and Subtyping of Food-Associated Pathogenic Microorganisms

Walter E. Hill and Karen C. Jinneman

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64.1 INTRODUCTION

Detection of disease-causing microbes in foods has always been a challenging undertaking. The United States (US) Centers for Disease Control and Prevention report the etiologic agent was confirmed in only 38% of the recognized outbreaks of foodborne disease in the US from 1983 through 1987.[18] Because pathogenic microbes may be present at very low levels, they can be difficult to detect in foods amid the indigenous microflora, and components of the foods may also interfere with their detection. Rapid techniques for the detection, identifi-

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cation, and enumeration of microbial pathogens present in foods are needed.

Standard methods for recovering bacteria from foods may include enrichment culture followed by streaking onto selective and or differential agar medium or direct plating onto these media. Individual colonies are identified by morphological characteristics. Confirming biochemical and/or immunological reactions and specific tests for microbial virulence factors usually requires between 2 and 5 days.

The invention of the gene probe and polymerase chain reaction (PCR)-based techniques permits the design and development of tests to rapidly detect and identify specific bacterial strains and/or virulence genes without the need to establish pure cultures. These methods have also lessened the need for virulence testing based on bioassays using animals. However, the implementation of these molecular biological techniques (usually designed for use in the research laboratory) to the routine analysis of foods is a formidable task and at present is a major area of activity.

Gene probe tests are based on the concept that single-stranded DNA molecules of complementary sequence will, under the appropriate conditions, reform the double helix. Therefore, if a piece of DNA is appropriately labeled, it may be used to detect complementary genes in the DNA of unknown organisms. If the labeled DNA probe becomes part of a double-stranded structure, it is concluded that the sample contained an organism with a particular genetic capability. There are several assay formats, and sensitivity is usually in the range of 10^4 to 10^6 copies of the target gene. Work on the genetic basis of pathogenicity of foodborne bacteria resulted in the development of gene probes targeted not only to specific genera and species but also toward particular strains that cause disease. Several reviews describe the use of gene probes in general[187,203,230] and their use to detect foodborne bacteria.[141,147,150,370,382]

The PCR is a procedure for the in vitro amplification of specific segments of DNA.[251,301,302] A millionfold increase of the target region usually can be achieved to permit the very sensitive detection (and possibly the quantitation) of specific genes. Introduced in 1985, this technology has been applied in a broad range of disciplines ranging from anthropology to forensics and zoology. The use of PCR to detect foodborne pathogens has been reviewed.[148,179]

Many of the difficulties in using gene probes and PCR-based methods for the analysis of food samples are in the area of sample preparation; that is, releasing DNA from colonies and from food matrices and the removal of inhibitors of DNA amplification.

Progress in the sequencing of nucleic acids and the wide range of potential uses for gene probes helped stimulate the development of methods to synthesize oligonucleotides. PCR-based tests would be laborious to develop and difficult to perform were it not for the general availability of defined-sequence oligonucleotides. In addition, many of the genetic analyses of bacterial virulence factors created a solid foundation for the use of gene probes and PCR applications in that the nucleotide sequences of a variety of virulence genes and taxonomic markers were determined. Conveniently, oligonucleotides used as probes often could serve as primers. The PCR offers a new level of sensitivity and speed for the detection and identification of microorganisms, but the use of gene probes following direct plating or enrichment culture can yield more reliable quantitative estimates of viable pathogens in food samples.

The focus of this chapter is gene probe and PCR-based methods of testing foods for the presence of hazardous microorganisms and not on the use of probes and PCR as research tools. The chapter will outline general mechanisms and formats and give examples of specific applications for foodborne microbial pathogens. Genome characterization methods such as pulsed-field gel electrophoresis (PFGE) and ribotyping, which are becoming more important for the subtyping of strains for epidemiological purposes, will also be described.

64.2 GENE PROBES

Gene probes are labeled DNA fragments and are used for the detection of particular genes by hybridization. The gene probes are labeled so that one may determine whether they have formed hybrids with the sample DNA.

64.2.1 DNA Hybridization

The DNA molecule usually exists as a double-stranded helix held together by hydrogen bonds between adenine and thymine and between guanine and cytosine. The strands can be separated (denatured) by increasing the pH or the temperature. When the pH or the temperature is lowered, the strands will reform the double helix if strands contain similar sequences. If the strands reforming the double helix originate from different sources, the resulting molecules are called hybrids and the process is termed hybridization. When a gene probe is found stably associated with the target DNA, it is concluded that the target DNA contains the same information as that encoded in the probe.

64.2.2 Hybridization Conditions

A major factor in controlling the specificity of hybridization is the temperature at which it is performed. When the temperature is too low, the gene probe will reform the double helix with regions that are not exactly complementary (low stringency), causing false positive results. At too high a temperature, even perfectly matched hybrids will have difficulty remaining in the double-stranded state, and false negative reactions may be observed. Normally, a suitable hybridization temperature is determined empirically but there are a number of algorithms for estimating this temperature. Salt concentration also plays an important role: generally, increasing the salt concentration reduces the stringency; an increased temperature will be required to maintain the stringency.

The concentrations of the gene probe and the target nucleic acid affect the time required for hybridization. At higher concentrations, the reaction will proceed more quickly but when concentrations are too high, self-annealing of the probe or of the target will decrease reaction sensitivity. However, when oligonucleotide probes are used, all the molecules are identical and hybridization of probe molecules with themselves is not a problem. Generally, reactions using synthetic oligonucleotide probes can proceed more rapidly than reactions using long gene probes because higher concentrations can be used and the small oligonucleotides have a faster rate of diffusion.

64.2.3 Hybridization Formats

Several hybridization formats are available. The minimum requirements are that the target DNA is exposed, denatured, and able to be hybridized and that the hybridized probe:target complexes can be identified.

A solid support can be used to anchor either the target DNA or a probe and provide an easy means to remove nonhybridized components prior to detection. A common format, colony hybridization (Figure 64–1), involves the transfer of colonies from an agar plate medium to a membrane. Cells are lysed and the DNA released, denatured, and affixed to the membrane. The gene probe hybridizes with the immobilized target DNA and the unreacted, labeled probe molecules are removed by rinsing. The presence of probe is detected by a method appropriate for the particular probe label. Colonies that contained the target DNA will be indicated by corresponding dots on the membrane. Nitrocellulose filters were first used with ^{32}P-labeled probes and required baking to fix the DNA to the surface.[134] Then Whatman 541 filters were introduced, on which the DNA can be adhered by air drying; these filters also had the advantage of not becoming brittle.[229] Positively charged nylon membranes are reported to be optimal with nonisotopic probes because the nonspecific background signal is reduced.[72] The DNA can be fixed to these filters by either baking or ultraviolet (UV) crosslinking. The transferred colonies often are lysed and the DNA denatured by exposure to alkali solution[134,229] or by the use of microwaves.[74] Cell lysis may also be accomplished by using proteinase K[311] or sodium dodecyl sulfate (SDS).[297] Excess colony debris can be removed mechanically from the membrane surface by gently rubbing it with a tissue soaked in 5-fold concentrated standard saline citrate (5XSSC) solution to reduce background signal especially with digoxigenin-labeled probes.[145]

Colony hybridization may not be sufficiently sensitive for detection of pathogenic microorganisms in foods; for example (1) if the level of the pathogen is too low to be detected by direct plating, (2) when high levels of indigenous microflora are present (usually above 10^6 to 10^7 cells g^{-1}), or (3) if the target genes are on plasmids, which may be lost during enrichment.[149,185,395] Other methods using target DNA immobilized on a solid support are dot blots of lysed and denatured cells from a liquid suspension or amplified PCR product. In a Southern blot format, specific DNA fragments are separated by gel electrophoresis, denatured, and fixed onto a membrane.[322]

In some formats, one gene probe is fixed to a solid surface, such as a dipstick or the bottom of a microtiter dish, and used to "capture" the denatured target DNA. The Gene-Trak® products for *Listeria* or *Salmonella* use a dipstick coated with a "capture" probe targeted to specific regions of the 16S rRNA gene from a lysed cell suspension (Figure 64–2). Because the target is present in multiple copies per cell, the sensitivity of the test is greater than those depending on a single target copy. This increased sensitivity allowed the development of a colorimetric detection system of sensitivity equivalent to the

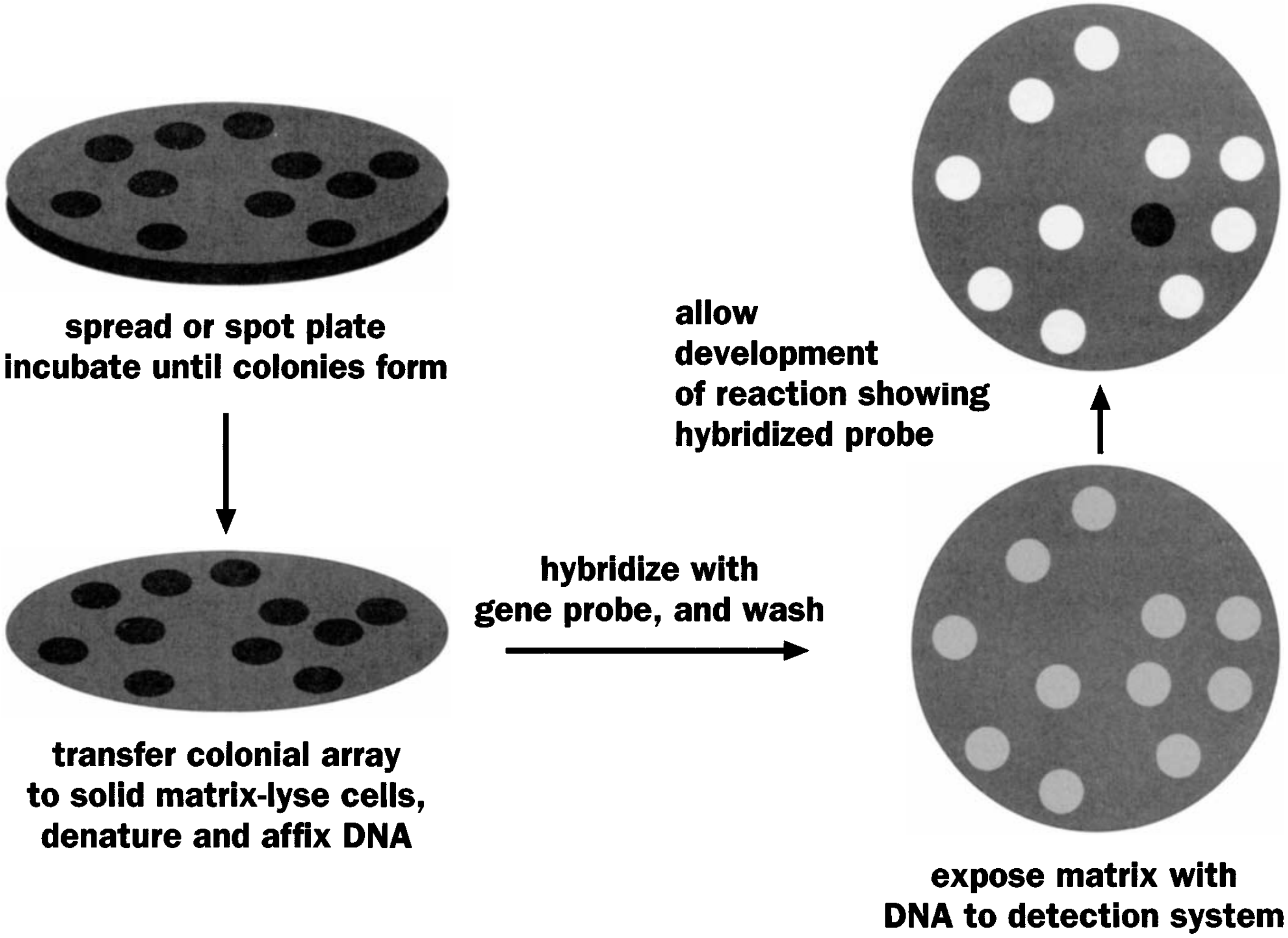

Figure 64–1 DNA Colony Hybridization

isotopic assay. A second probe, a reporter probe, labeled with fluorescein isothiocyanate (FITC) hybridizes with the captured target DNA and is detected by chromogenic methods.[69,192,194]

Hybridization formats also have been developed where all components are in solution. One such system uses two gene probes (each with a unique label) that must be physically close to each other to emit a signal. These two labels will be in proper geometric relationship only when they are bound to the target DNA. Thus, hybridization reactions can be followed in real time with no interference from unbound probes.[53] Another format, available as a commercial kit (AccuProbe *Listeria monocytogenes*) uses a DNA probe complementary to the rRNA of the target organism. The rRNA is released from cells so that it can form a stable DNA:RNA hybrid with an acridinium ester-labeled DNA probe. The unhybridized probes are selectively inactivated by differential hydrolysis. The addition of an alkaline H_2O_2 solution allows the detection and quantitation of light emission from the acridinium esters protected by linkage to the DNA:RNA hybrid.[257]

64.2.4 Labeling of Gene Probes

During the early development of gene probes, radioactive labels, usually ^{32}P, were used to achieve the necessary sensitivity. The label was detected by autoradiography (X-ray film) or by Geiger-Mueller or scintillation counting. The use of radioactivity hampered the widespread implementation of these techniques in laboratories concerned with routine testing. Subsequently, nonisotopic probes were developed that are stable for long periods (1 to 2 years). Large (microgram) amounts of DNA can be labeled at a time, allowing for multiple tests from the same lot of labeled probe. Many nonisotopic re-

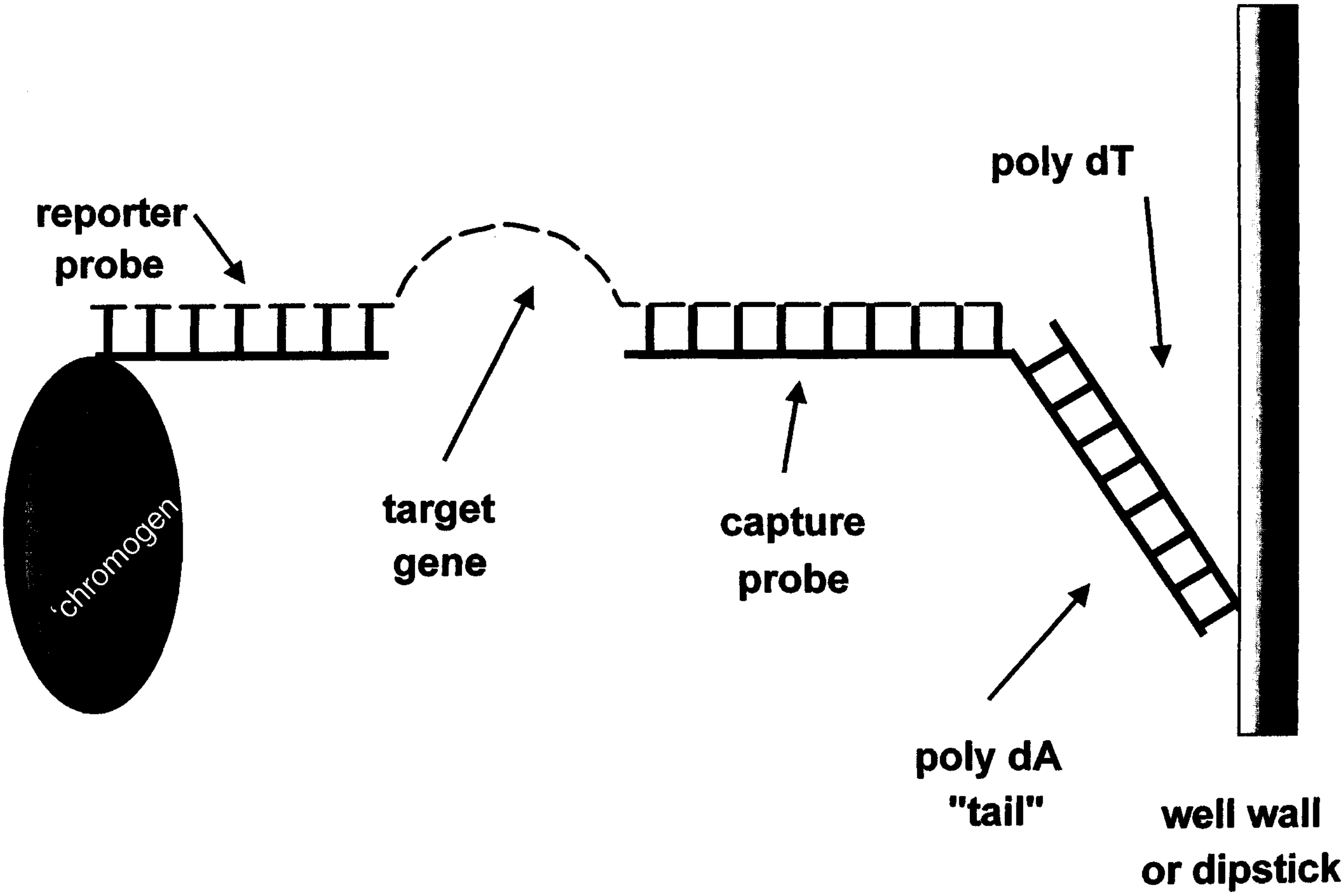

Figure 64–2 Sample Two-probe DNA Hybridization System

porter molecules are incorporated into commercially available kits. The disadvantages have been due primarily to interference from endogenous substances, which can reduce the sensitivity of the probe.

Nonradioactive indicator systems can use either direct or indirect detection. In the direct systems, bifunctional conjugates are prepared by covalently linking the oligo- or polynucleotide probe directly with the signal-generating reporter molecule. The advantage here is that only a single reaction is required for detection; however, the reporter molecule must retain its activity during the hybridization procedure. Indirect systems link a modified group to the oligo- or polynucleotide probe. After the hybridization is complete, a molecule recognizing the modified group and covalently linked to a signal-generating reporter molecule is used to detect the hybridized probe.

The most common nonradioactive nucleic acid probe-labeling systems for the detection of foodborne pathogens or PCR-amplified products have used either biotin[58,92,227,297,348] or digoxigenin.[145,164,167,188,190,191,227] Chemiluminescence is useful for quantitative detection of PCR products.[67,83,392] The direct incorporation of horseradish peroxidase[36,72,145] or alkaline phosphatase[163,259,269] to oligonucleotide probes also has been used for direct hybridization assays. The choice of nonradioactive labels depends on whether the probe is a fairly long (100 to 1000 bp) cloned fragment or a short oligonucleotide, whether the probe is DNA or RNA, and the intended hybridization format (eg, Southern blot, dot or slot blot, colony lift analysis, liquid format, or target capture). The following methods are used for labeling gene probes.

Random Primers. This method is appropriate for labeling DNA probes 100 to 10,000 bp in length. The template DNA is first purified, linearized, and denatured. Short (6 to 8 bp) random nucleotide segments, some of which are labeled with a modifying group or reporter molecule, are annealed to the template DNA to produce short regions of double-stranded DNA followed by single-stranded stretches. The Klenow fragment of DNA polymerase (the large fragment of the *E. coli* DNA polymerase that does not have 5' → 3' exonuclease activity) synthesizes a complementary strand incorporating the modified nucleotides. The reaction is terminated and labeled probe is precipitated and denatured for use in hybridization.[285]

Nick Translation. Template DNA is nicked with DNase; *E. coli* DNA polymerase I is used to add labeled nucleotides to the 3'-hydroxyl termini created by nicks in the double-stranded DNA. The 5' → 3' exonucleolytic activity of DNA polymerase I removes nucleotides from the 5' side of the nick and simultaneously continues to add nucleotides to the 3' termini.

3'-End Oligonucleotide Labeling of Oligonucleotides. Oligonucleotide probes generally are defined as probes of 50 bp or less. Automated DNA synthesizers allow the production of large amounts of oligonucleotides that can be labeled in several ways. Terminal transferase labels the 3'-end by adding a tail or a single dideoxynucleotide containing a modified group or reporter moiety. The 3'-end labeling reaction does not elongate past the single dideoxynucleotide.[349] A 10 to 100 bp tail can be added to the 3' end of an oligonucleotide using terminal transferase and a mixture of labeled and unlabeled nucleotides.[38,289] The long tail may improve test sensitivity but may result in nonspecific binding during hybridization.

5'-End Oligonucleotide Labeling. Oligonucleotides labeled at the 5' end leave the 3' end free to act as a primer in amplification reactions, yielding labeled products.[4]

Labeling RNA by Transcription. Linearized template is cloned adjacent to a RNA polymerase promoter site. RNA polymerase synthesizes a transcript using modified precursors to incorporate reporter molecules.[240,247]

Preparation of Labeled Copy DNA (cDNA). The avian myoblastosis virus (AMV) reverse transcriptase can synthesize a cDNA probe from a heat-denatured RNA template by using labeled deoxyribonucleoside triphosphates (dNTPs).[335]

Labeling DNA by PCR. A PCR using primers that bracket the target area of DNA is run with labeled dNTPs, which are incorporated into the product.[91,121]

Labeling by Photo-Activation. Nonenzymatic photoreactions covalently incorporate a modifying group into probe DNA via illumination at a specific wavelength. Photodigoxigenin, a digoxigenin molecule linked via a hydrophilic spacer to an azido phenyl residue, or psoralen biotin have been used.[250,316]

Other Nonenzymatic Labeling. Both single-stranded DNA and RNA can be labeled nonenzymatically with horseradish peroxidase that has been complexed with a positively charged polymer. The nucleic acid probe and the horseradish peroxidase are attracted by charge and glutaraldehyde is added to form chemical crosslinks.[284] The hybridization procedures must not inactivate the peroxidase.

64.2.5 Detection of Gene Probes

As mentioned above, detection of radioactive gene probes was originally done by autoradiography and occasionally by scintillation counting or Geiger-Mueller tube counting. However, detection methods for nonisotopic labels are more varied.

Depending on the type of probe label, either a direct or indirect detection procedure is used. Affinity-based reporter molecules such as biotin or digoxigenin are detected via indirect methods. Following hybridization, antibodies covalently linked to enzymes such as alkaline phosphatase or horseradish peroxidase (in the case of biotin, streptavidin is used instead of an antibody) are introduced and bind specifically to probes. Unbound molecules are washed away. Then, colorimetric or chemiluminescent substrates are added. Colorimetric substrates often consist of 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT) solutions where results are developed in place. Chemiluminescent dioxetene substrates are cleaved by alkaline phosphatase to give a localized light emission detected by exposure of X-ray film. This improves sensitivity and multiple exposures can be made. In a study comparing methods for the quantitation of PCR-produced DNA, electrochemiluminescence was 10 to 100 times more sensitive than ethidium bromide fluorescent staining.[392]

64.3 THE BASIC POLYMERASE CHAIN REACTION (PCR)

64.3.1 Description

Several review articles and books describe the mechanism of the PCR and other amplification reactions.[132,382] More than 10,000 papers per year are cited in MEDLINE.[148]

PCR is a method for amplifying a given region of DNA by using a pair of specific oligonucleotides that bracket that region and serve as primers for polymerase to initiate DNA replication (Figure 64–3). This section is copied repeatedly. The template DNA strands are separated by heating and then are cooled to allow primers (at a concentration about a billion-fold greater than the template) to reform the double helix (anneal) with the template strands. The temperature at which this occurs is critical for controlling the stringency of primer binding to the template, a crucial factor in determining the specificity of the amplification reaction. Usually a third temperature is used to allow copying of the template strands (the primer extension step) to occur at the optimum temperature of the DNA polymerase, but there are many variations. The newly synthesized DNA strands serve as templates for subsequent rounds of replication; therefore, the amount of DNA created theoretically increases by a factor of 2 at each cycle. During the second round of synthesis, product strands that terminate in primer sequences arise; these defined-end molecules increase exponentially in subsequent cycles, becoming the predominant product. These molecules can be detected and quantitated by gel electrophoresis followed by ethidium bromide staining, ultraviolet light illumination, and colorimetric reactions or chemiluminescence. The high specificity possible with PCR arises because it is a hybridization method using two gene probes; the amplification is achieved by repeating the hybridization 30 to 40 times.

Initially, PCR was performed by moving tubes manually among three heating blocks or water baths and, because the DNA polymerase was inactivated by the denaturation step, fresh enzyme had to be added during each cycle. To increase the usefulness and convenience of PCR, the technique was automated. While a programmable heating and cooling block was relatively simple to develop, a critical feature was the use of a thermally stable DNA polymerase from the thermophilic microbe *Thermus aquaticus* (*Taq* DNA polymerase).[43]

All components necessary for PCR analyses are now available commercially, including reaction buffer, $MgCl_2$, the four deoxyribonucleotides (dATP, dTTP, dCTP, and dGTP), and several thermotolerant DNA polymerases. Oligonucleotide primers specific for a selected genetic target sequence can be prepared commercially according to customer specifications. Assembling and using individual PCR components allow analysts to optimize a system for their specific needs. However, a complete PCR kit for amplification of unique *Salmonella* gene sequences from preenriched food samples of fluid or dried milk, chicken, turkey, beef, and pork has been introduced (DuPont, Wilmington, DE).[165,169] The primers, polymerase, and nucleotides are lyophilized into a tablet at the optimum concentrations and packaged within tubes for PCR amplification. The DNA from the enriched food sample is prepared with lysis solution and added to the PCR amplification tubes. Control tubes containing *Salmonella* DNA are used to detect DNA replication inhibitors. The amplified product is detected by gel electrophoresis. Kits for the PCR detection of *E. coli* O157, *Listeria* species, and *L. monocytogenes* have become available.

64.3.2 Sensitivity

It is important to determine the sensitivity of PCR-based assays for the detection and identification of foodborne microorganisms; however, no standard has been adopted for the determination and reporting of sensitivity. If PCR-based methods are to be adopted, they will most likely need to receive the Association of Official Analytical Chemists International's (AOAC) approval or the equivalent and, therefore, will be compared with standard methods. This will require that sensitivities be calculated as the number of bacterial cells detected per 25 g of sample. Now, many authors report the sensitivity in picograms of DNA; to evaluate the relevance of these data, they should be converted to number of microorganisms, a more biologically meaningful quantity. If most bacteria have a genome approximately the size of that of *Escherichia coli* (about 5 fg), then 1 pg of DNA is equivalent to about bacterial 200 genomes, and 1 ng represents about 2×10^5 bacterial cells.

64.3.3 Fidelity

For PCR-based detection methods, high-accuracy replication of DNA may not be required. The primary determinative step of these assays is whether the amplified product is of the expected size. During test development, the genetic information of the amplicon may be verified by restriction endonuclease digestion, further amplification with nested primers, hybridization with a gene probe targeted to a region within the amplicon, or by nucleotide sequencing. However, during routine use of these PCR

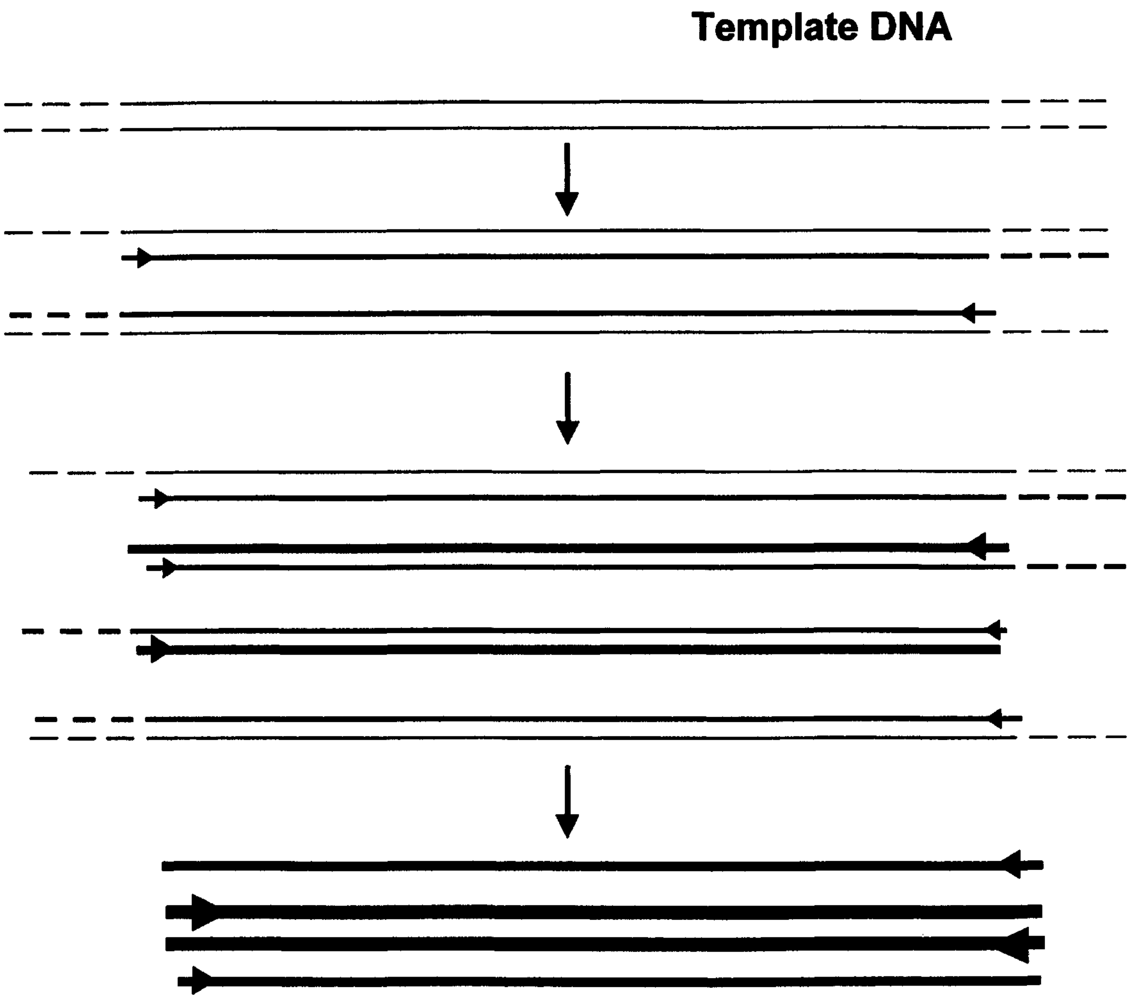

Figure 64–3 The Polymerase Chain Reaction. Segments with arrows represent primers. The thicker lines represent more recently synthesized strands. Courtesy of R. Myers.

tests, further characterization of the amplicon usually is not carried out.

For cloning or for collecting nucleotide sequences for strain comparison, high-fidelity DNA replication is more important. Several thermostable enzymes have putative proofreading activity (*Pfu*, Deep Vent, *UlTma*) and reduce errors during replication. Replication accuracy is influenced by several factors, including the insertion of the wrong nucleotide; proofreading by a 3' → 5' exonuclease can lead to removal of mismatches followed by repair extension. For the *Taq* polymerase, the error rate per base pair ranges from 1.1×10^{-4} [341] to 2.0×10^{-5}.[228] Deletion of the N-terminal end of this enzyme reduced the error rate to about 5.1×10^{-5}, an improvement of about a factor of 2.[16] Similar results were obtained using a yeast transformation assay.[106] These rates appear to be considerably higher than those estimated for in vivo replication and, when propagated over 30 to 40 amplification cycles, could allow significant errors to accumulate. If ambiguous results are obtained, it is recommended that nucleotide sequences should be determined from DNA independently amplified from several lots of starting material.

64.3.4 Selection of Primers

Computer software has been developed to search for sequences that fulfill particular criteria,[315] but the final test of primer effectiveness often is determined empirically. To develop a test with high specificity, primer sequences should be unique and targeted to a single region. The similarity of a proposed region to other sequences can be determined from databases such as GenBank® or that compiled by the European Molecular Biology Laboratory (EMBL) by using specially designed software.[6,270] This process does not ensure that the primer regions are unique but it does provide some confidence that the sequence(s) is not common, or that it does not share sufficient homology with other regions to be nonspecific.

Another important factor is the estimated denaturation ("melting") temperature of the template-primer hybrids and the selection of the appropriate annealing temperature for the PCR. Again, software can assist in finding segments with suitable T_m (melting) or T_d (dissociation) temperatures;[156,226,294] such programs usually check for hairpin loops, primer self-complementarity, and complementary regions between pairs of primers to reduce the formation of primer dimers. Primers with GC rich 3' ends (a "clamp") appear to establish more stable sites for the initiation of polymerization; however, a run of three or more G or C bases in the 3' position could stabilize nonspecific primer annealing. In the selection of primer sequences, characteristics of the amplified region such as size or the location of restriction endonuclease sites should be considered.

64.3.5 Optimization of Reaction Conditions

Although the basic PCR is fairly simple, several parameters have a major effect on its specificity and efficiency.[299,300] Divalent cation concentration, pH, primer sequence and concentration, hybridization stringency (as influenced by annealing temperature), number of cycles of replication, time of elongation cycle, and the size of the amplified fragments are important.[71,158] The annealing temperature and the salt concentration affect hybridization stringency. If the annealing temperature is too low, hybridization stringency is reduced; nonspecific priming may occur and several regions may be amplified. The temperature of annealing is particularly important during the early replication cycles as disparate regions may be amplified.[291] To minimize false priming during the first cycle, the polymerase can be separated physically from the other reaction components by wax. This separation prevents extension below the annealing temperature because the wax melts during the initial denaturation step allowing the polymerase to combine with other components.

A further procedure to prevent nonspecific priming uses an initial annealing temperature above that required for primer binding; the temperature is lowered for each successive cycle until a product is formed.[84] Heating before amplification may also increase the specificity of the reaction.[71] By holding the reaction at a higher temperature, the annealing of primers with each other (primer dimer formation) may be reduced. This is important because primer concentrations should remain high compared with that of the template. Preamplification heating may encourage a higher stringency of primer annealing during the first cycle of elongation. If short regions of DNA are being amplified, a lower denaturation temperature may increase the amount of product.[389] A procedure for calculating annealing temperatures has been advanced.[295] Although algorithms for estimating annealing temperatures provide a starting point, empirical results usually govern the selection of parameters.

The PCR is influenced by divalent cation concentrations and, for each set of primers, the magnesium ion concentration should be optimized for priming specificity and amplification efficiency. For most sets of primers, 1 to 6 mmol $MgCl_2$ l^{-1} is the optimum range. Concentration artifacts range from no product to the generation of several amplicons, ostensibly caused by a decrease in the specificity of the enzyme for the primer-template hybrids. Template concentration can also have a pronounced effect: if too high, the large numbers of variable-length copies produced usurp precursors so that the target region will not be replicated efficiently, leading to a false negative result or smears of PCR product observed after gel electrophoresis. An excessive number of amplification cycles can also generate variable-length molecules and thus decrease the amount of the specific product.[26]

64.3.6 Prevention of Carryover Contamination

Completed amplification reactions may contain billions of copies of molecules that include complementary primer sequences on each end. Because a single region of template DNA can be amplified many million-fold, PCR products must be considered much like infectious agents that can "contaminate" future reactions; therefore, it is important to minimize cross-infection. Physically separate areas and liquid handling equipment can be used for setting up reactions and for analyzing amplified material. Aerosol-reducing pipet tips and positive-displacement micropipetors help to reduce cross-contamination. Liquid handling should be kept to a minimum by making master mixes of reagents to further reduce the possibility of adulterating reactions.[205] Ultraviolet light will crosslink any stray DNA and prevent it from being replicated.[62] Another ingenious method to reduce carryover of amplified product into subsequent reactions uses uracil incorporation. Ensuing reactions are treated with uracil DNA glycosylase that cleaves uracil-containing DNA;[223] this DNA cannot function as template. The enzyme is inactivated by heating before carrying out amplification. Rys and Persing[296] compared three methods to inactivate amplified product and eliminate carryover contamination: post-PCR product cross-linking with isopsoralen, the treatment of dU-containing products with N-glycosylase, and hydrolysis of PCR products incorporating 3′ ribose-containing primers. These methods were all capable of inactivating 10^4 to 10^9 copies of amplified product.

64.4 GENERAL ASPECTS OF APPLICATION OF THE PCR TO DETECTION OF MICROORGANISMS IN FOOD SAMPLES

64.4.1 Preparation of Samples

The application of PCR to the detection of microorganisms in food requires efficient template preparation. It appears to be much easier to prepare DNA adequate for hybridizations than to prepare efficiently amplified template DNA. Food samples vary in composition and consistency; they may have low numbers of target organisms and high numbers of other microorganisms. Thus, they more often present sample preparation problems than do clinical samples. An internal control for amplification is important when samples may contain PCR inhibitors.[375] A conserved region of the 16s rRNA gene serves this purpose well.

Enrichment culture is often used before the PCR. Although an enrichment step lengthens the time of analysis, it dilutes inhibitors in the food and increases the number of target organisms.[7,23,109,119,152,174,321,369] Enrichment may induce the loss of genetic elements, particularly those on plasmids; therefore, it may be advantageous to omit or minimize enrichment.[206] Centrifugation and washing techniques can concentrate target organisms and remove some PCR inhibitors.[65,282,366,378] Filters and flow cytometry can concentrate bacteria from water and other samples.[175,303] Another strategy to concentrate organisms is antigen capture. For example, immunomagnetic separation (IMS) uses super-paramagnetic particles or beads coated with antibodies against surface antigens to "capture" specific target microbes and remove and concentrate the organisms. Both polyclonal and monoclonal antibodies have been employed. The antibodies can be attached to the beads either directly or indirectly, via primary antibodies covalently attached to the beads. IMS techniques are reviewed elsewhere[263] and have been used to detect a variety of foodborne pathogens including *L. monocytogenes*,[109] enteropathogenic *E. coli*,[157] *E. coli* O157,[174] *Salmonella*,[110,372,373] *Shigella dysenteriae* type 1 and *Shigella flexneri*,[161] *Yersinia enterocolitica*,[183] and *Cryptosporidium* oocysts.[175]

PCR inhibitors are a significant problem in many types of samples. Various strategies have been used to overcome their effects. For example, trace amounts of inhibitory humic substances in soil samples[346] have been removed by gel filtration.[345] Chelex resin[52,180,198] and various commercially available columns are used to bind potential PCR inhibitors.

Once the target organisms have been concentrated and inhibitors removed, the DNA must be available in an amplifiable form. Boiling is often used to release DNA from microorganisms. Other methods include phenol-chloroform DNA extractions, enzymes (eg, proteinase K), sonication, heating with microwaves, and thermal shock.[272] Guanidinium isothiocyanate and other chaotrophic agents assist in the disruption of cells and release of the DNA.[131,152,180,233] Columns[7] and DNA precipitation[42,119,186,233] have been used to purify and concentrate DNA.

64.4.2 Detection of Viable But Nonculturable (VBNC) and Dead Organisms

Tests for microorganisms in food are concerned almost exclusively with viable organisms. Because PCR methods may amplify DNA from dead microorganisms,[237] there is a need to assess whether the target organisms are viable. Because dead cells may contain amplifiable DNA, incubation of a sample for a short period and demonstration of an increase in product amplified by PCR has been used as evidence of the viability of target organisms.[206] Appropriate controls are necessary to detect the presence of any amplification inhibitors.

VBNC cells of some pathogens[290] might pose a health hazard if not detected by culture, but conflicting results exist regarding PCR detection of VBNC cells.[22,162,181] The effect of stress treatments such as starvation, acid, heat, H_2O_2, or desiccation on the detection of *L. monocytogenes* and enterotoxigenic *E. coli* (ETEC) by PCR have been studied.[237] The type of stress to which cells were exposed affected the relationship between plate count and detection by PCR. Exposure to acid or to H_2O_2 resulted in a rapid reduction of plate count and detectability by PCR; however, starvation and heat (including exposure to 100° C temperatures and autoclaving) that caused a rapid reduction in plate count did not result in a rapid loss of detectability by PCR. *Vibrio vulnificus* can be difficult to isolate from the marine environment during the cooler months when it may be in a VBNC state; however, this state can be reversed by resuscitation. When *V. vulnificus* cells were treated in the laboratory to render them viable but unable to form colonies on plating, they were more difficult to detect by PCR than predicted.[41,256] This may have been because the DNA had accumulated single-stranded nicks, rendering it a poor template for PCR but not greatly affecting viability.

Another way to demonstrate viability of the target organism is by using mRNA, which has a shorter functional half-life than DNA, as a template (see the section on reverse transcriptase [RT] PCR). When purified mRNA was used to generate cDNA in an RT-PCR, for the *ctx* gene of *V. cholerae*, no amplification was observed when nonviable cells were tested.[246] The method by which *Giardia* cysts were killed affected detection by RT-PCR.[231] Freezing prevented PCR amplification of mRNA, but heat-treatment or exposure to monochloromine did not.[231] The amount of total RNA and giardin mRNA recovered from live cysts increased after excystation. Therefore, either measuring total RNA or performing RT-PCR for giardin mRNA before and after excystation can be used to distinguish between live and dead cysts.[231] Similarly, *Cryptosporidium* oocysts could be detected by induction of excystation to provide intact, amplifiable DNA and demonstrate viability.[103]

In some instances, the PCR amplification of DNA in the absence of viable cells could be important. PCR detection of staphylococcal enterotoxin genes might predict the presence of the enterotoxin(s) in samples although thermal processing may have killed the vegetative cells.[378]

64.4.3 Detection of PCR Products

Detection is based primarily on the observation of an appropriately sized product visualized by UV illumination of ethidium bromide-stained gels. PCR assay sensitivity and specificity have been enhanced in many protocols by employing more sensitive methods for the detection of amplification products. Methods that are more sensitive usually involve gene probes to the amplified region. The amplified product may be spotted onto a solid support, such as a membrane, and followed by gene probe hybridization.[21,29,42,51,77,110,157,167,175,207,261,268,320,359] Alternatively, a probe can be affixed to either a membrane or a microtiter well to "capture" the PCR amplicons that are then detected with a second "reporter" probe.[52,118] A "reporter" molecule such as digoxigenin-11-dUTP[164] or a biotin-labeled primer[320] can be incorporated into the amplicon during the PCR process.[227] When these amplicons are captured, they can be detected directly without the use of a second "reporter." When both biotinylated primers and digoxigenin-dUTP are incorporated during PCR, the amplification products can be captured by streptavidin-coated microtiter wells and detected by antidigoxigenin-alkaline phosphatase conjugate.[227]

An oligonucleotide ligation assay (OLA) can detect PCR amplified products from *Salmonella*.[325] Following amplification, the product was denatured and oligonucleotide probes targeted to adjacent regions were hybridized to the amplified DNA. One probe was biotinylated on the 5' end and referred to as the "capture probe"; the "reporter probe" was labeled with digoxigenin on its 3' end. Ligation between the two probes was accomplished if there was complete complementarity. The ligated probe complex then was captured on streptavidin-coated microtiter wells where the reporter molecule was detected after the addition of a chromogen-substrate complex.

An alternative approach uses a fluorescently labeled internal probe and a quencher dye coupled to individual nucleotides near to each in the primer; fluorescence is reduced because these moieties are close. This internal probe anneals to the template but the *Taq* polymerase's 5' → 3' nuclease activity digests the internal probe during the extension step of each cycle. Therefore, the quenching dye is removed from its nearby location so fluorescence is increased and is proportional to the amount of the PCR product.[17]

Blais and Phillippe[36] describe the use of an unlabeled RNA probe complementary to the amplified *Listeria hlyA* fragment and a polyclonal anti-RNA-DNA IgG antibody peroxidase conjugate to increase the sensitivity of detection of amplified PCR product compared with agarose gel electrophoresis. This approach was refined further by using one PCR primer containing a T7 RNA polymerase promoter.

The amplified product was transcribed using T7 RNA polymerase and the RNA transcript captured by a DNA probe immobilized on microtiter wells. An immunoenzymatic assay with anti-RNA-DNA antibodies was used to detect as little as 0.15 ng of amplified product representing a 10-fold improvement over gel electrophoresis.[35]

64.5 MODIFICATIONS OF THE BASIC PCR METHOD

Several modifications of the original PCR procedure have been reported that might be applied to testing foods.

64.5.1 Multiplex PCR

Multiple sets of PCR primers are used concurrently within the same reaction tube to amplify simultaneously several regions of a target genome.[24,25] Some interaction may occur among the primer sets so primer concentrations might have to be adjusted to produce reliable yields of all the PCR products.

64.5.2 Nested PCR

It is possible to dilute the impact of template contamination with previously generated PCR products and reduce the effects of PCR inhibitors by carrying out two serial PCRs. When the first PCR is completed, between 0.01 and 1 µl of the reaction mixture is used as template for a second PCR. A second set of primers targeted to sequences on the 3' sides of the original primers generates a second, slightly shorter product.[279] A modification of the multiplex and nested primer PCR involves three primers targeted to the same gene to provide additional confirmation.[51] For example, the two "forward direction" primers and one "reverse" primer may result in two amplification products, reducing the risk of nonspecific amplification.

64.5.3 Inverse PCR

Ordinarily, the nucleotide sequence or the restriction endonuclease sites of the region to be amplified are known. When primers are made so that their 3' ends extend away from each other and the template DNA is cleaved with a restriction enzyme distal to this region and then circularized by DNA ligase, the unknown genetic sequences outside the region can be amplified and sequenced.[260,343]

64.5.4 Mismatch Amplification Mutation Assay (MAMA)-PCR

This method can distinguish between targets that differ by only one nucleotide.[56] Primers are constructed so that the 3' end of one of the primers contains a single mismatch with the allele to be detected and a double mismatch with the allele to be excluded. Thus strains of bacteria that differ by a single nucleotide in the amplified region can be detected and differentiated (Figure 64–4).[55]

64.5.5 Quantitative PCR

It is difficult to estimate the initial concentration of the template in the reaction from the amount of PCR product synthesized. A small difference in reaction efficiency can result in a substantial difference in the amount of the final product. One method to estimate the initial concentration of PCR template requires the concomitant amplification of a control molecule of known starting concentration and then a comparison of the final concentration of each product.[19,40,64,126,143,254] There is some uncertainty in this type of measurement, because there can be differences in amplification efficiency associated with the different primers or with the nature of the amplified region itself. This problem might be minimized by using the same primers to amplify a control region that would be distinguished from that of the unknown by differences in length or restriction endonuclease sites. The control templates could be added directly into a sample to ascertain whether PCR inhibitors are present by comparing the amounts of the products.[64]

Limiting dilution can be used to calculate the initial number of PCR templates in a sample.[326] This would determine the most probable number (MPN) of detectable PCR templates. A direct comparison of the MPN of microorganisms detected by a cultural method and the MPN of templates detectable by PCR can be made. (The statistics of MPN counts are covered in Chapter 60.)

64.5.6 Reverse Transcriptase PCR

When an RNA template (such as some viral genomes or mRNA) is to be amplified, a strand of DNA (cDNA) is made using RT and the "downstream," or "reverse" primer (Figure 64–5). The cDNA can then serve as a template for a standard PCR.

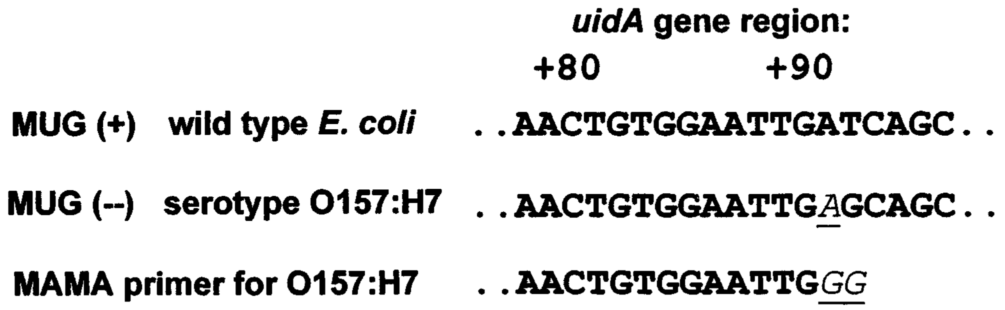

Figure 64–4 Mismatch Amplification Mutation Assay Used to Detect *Escherichia coli* O157:H7. The primer for amplification of *E. coli* O157:H7 DNA contains one mismatch at the 3′ end with the intended target, but two mismatches with the genotype to be excluded. This allows preferential amplification of the O157:H7 target over another template that differs by a single basepair.[55] *Source:* Adapted with permission from T.A. Cebula, W.L. Payne, and P. Feng, Simultaneous Identification of Strains of *Escherichia Coli* Serotype O157:H7 and Their Shiga-Like Toxin Type by Mismatch Amplification Mutation Assay-Multiplex PCR, *Journal of Clinical Microbiology*, Vol. 33, pp. 248–250, © 1995, American Society for Microbiology.

64.5.7 Degenerate Primers

The exact nucleotide sequence of a region may not be known or the identity of some nucleotide positions may be ambiguous (this might be the case if only the amino acid sequence of the encoded protein is available). In some cases, these difficulties can be overcome by synthesizing an oligonucleotide containing deoxyinosine in these positions[50,235] or by allowing more than one nucleotide at a particular spot creating a pool of mixed (degenerate) sequence molecules.

64.6 NON-PCR-BASED AMPLIFICATION METHODS

Three examples of such systems are described here briefly because they do not employ thermocycling and may, therefore, be simpler than PCR methods.

64.6.1 Self-sustained Sequence Replication (3SR) or Nucleic Acid Sequence Based Amplification (NASBA™)

This method uses a single temperature with a primer, an RNA template, and RT to generate a cDNA intermediate containing a bacteriophage T7 promoter (Figure 64–6).[94] RT makes a cDNA copy of the target RNA and the old template strand is degraded by RNaseH. The single-stranded cDNA serves as a template for the construction of a complementary strand. From this double-stranded molecule, T7 RNA polymerase synthesizes multiple transcripts of the region downstream from the T7 promotor. The transcripts are used as templates to construct additional cDNA molecules, which are then rendered single-stranded by RNaseH. The process continues at about 42° C; neither thermostable enzymes nor thermocycling is required. Amplifications of greater than 10^8-fold within 30 minutes have been observed.

64.6.2 Ligase Amplification (Ligase PCR)

The second term is a misnomer because the amount of product increases by joining two oligonucleotides instead of by synthesis of new strands by a polymerase. Two oligonucleotides are chosen to be complementary to adjacent regions of the targeted nucleotide sequence. When the target is present, the oligonucleotides will be positioned correctly for joining by DNA ligase (Figure 64–7).[255] When two pairs of oligonucleotides are used, one pair complementary to each strand, the number of ligated molecules will double each cycle; each molecule serves as a site for the alignment of a pair of oligonucleotides. This assay detects templates differing by only

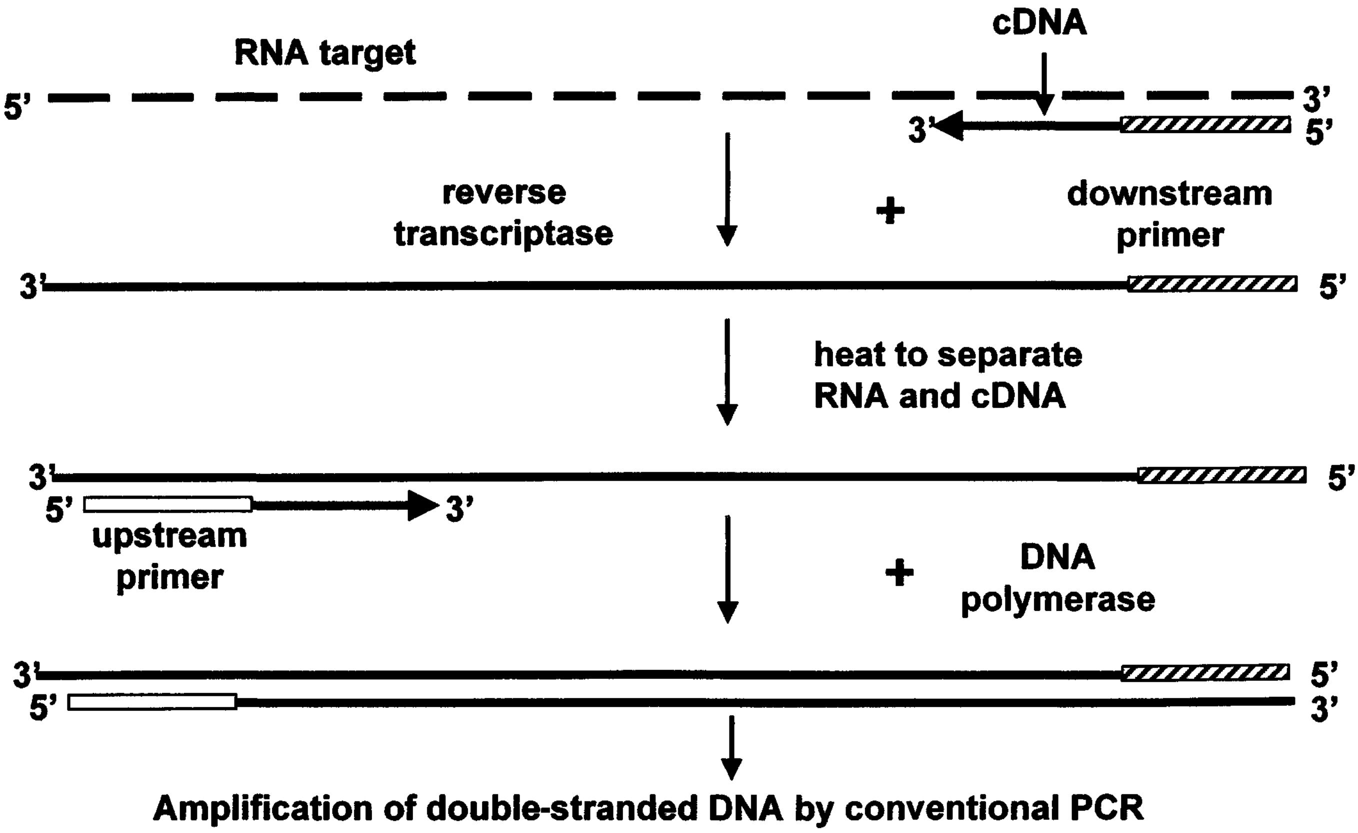

Figure 64–5 Reverse Transcriptase PCR

a single base pair when this site is located at the 3' end of the upstream primer.[15] It discriminates between *L. monocytogenes* and other *Listeria*.[374]

64.6.3 Qβ Replicase

The RNA polymerase from bacteriophage Qβ replicates RNA.[46,135,239] A detection probe, composed of a region complementary to the target and a region that can be copied by Qβ replicase, is hybridized to the target. The unbound probe is removed by hybridization to a capture probe attached to a solid support and complementary to a region amplified by the replicase. After the addition of Qβ replicase, the presence of the target nucleic acid is indicated by an increase in RNA.[202]

64.7 DETECTION OF FOODBORNE MICROBES

This section will discuss some examples of the use of genetic techniques to detect microorganisms in food. For further examples of the application of these methods, chapters on specific foodborne pathogenic microorganisms should be consulted.

64.7.1 Bacteria

Aeromonas spp. Members of this genus produce a variety of extracellular hemolysins and cytotoxins; aerolysin appears to be an important virulence factor (see Chapter 38).

Aerolysin is produced by some strains of *A. hydrophila* associated with human disease; the (*aer*) gene can be detected by PCR,[276] although its presence may not always correlate with strain virulence.[59] Similarly, Baloda et al.[14] targeted the β-hemolysin gene. Positive *A. hydrophila* strains were also hemolytic and toxic to tissue culture cells; however, hemolytic *A. sobria* and nonhemolytic *A. hydrophila* were negative by this PCR test. A 16S rDNA PCR assay detected aeromonads and, subsequently, primers were used to specifically detect *A. hydrophila* and *A. veronii*.[86] The gene for a cytolytic enterotoxin shares some similarity to *C. perfringens* enterotoxin and *L. monocytogenes* listeriolysin.[60]

Bacillus cereus. The gene encoding the enterotoxin, *bceT*, of *B. cereus* has been sequenced and may serve as the target of a PCR-based detection sys-

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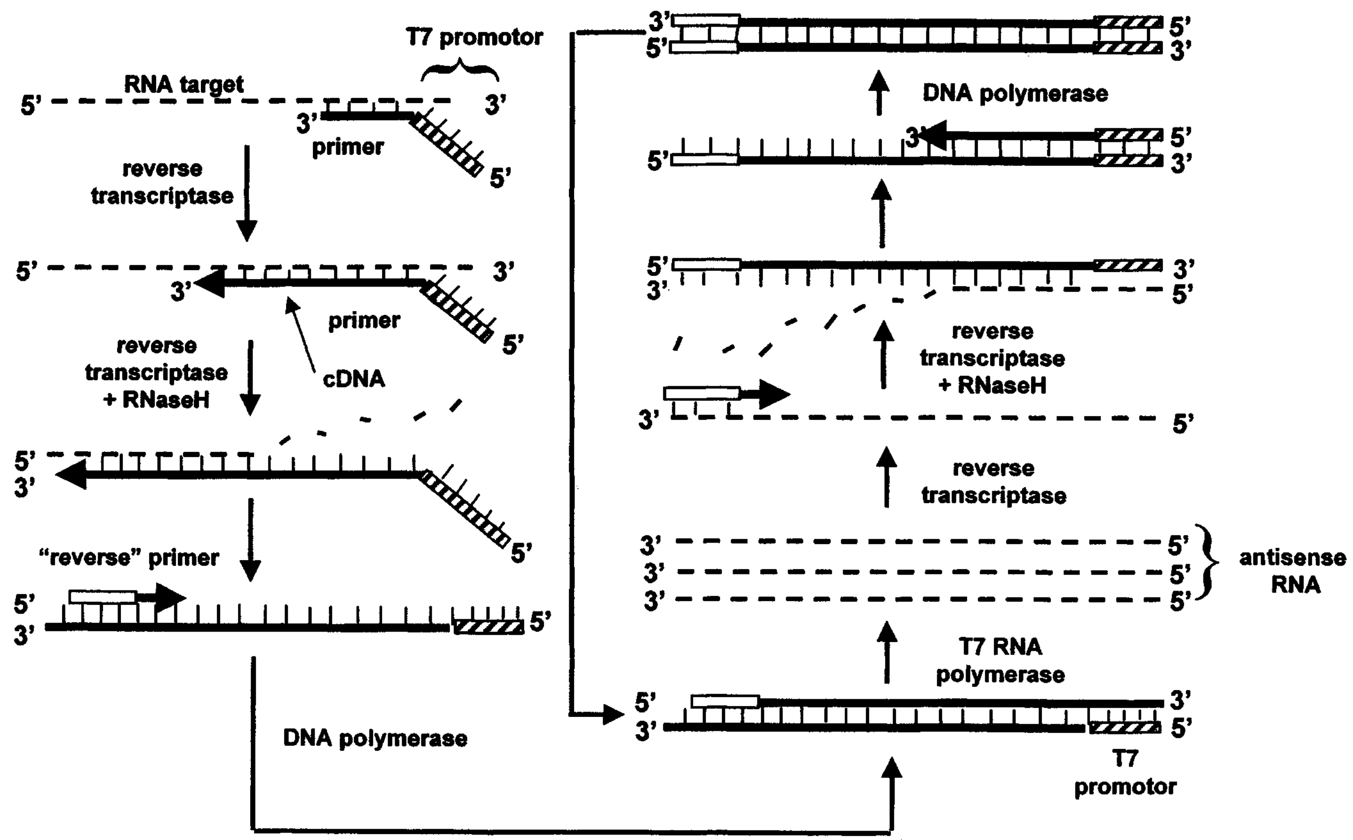

Figure 64–6 Self-sustained Sequence Replication (3SR)

tem.[3] Cereolysin AB, a cytolytic protein responsible for hemolysis, has been targeted with oligonucleotide probes.[310] Although there are no reports of these procedures being applied to foods implicated in disease outbreaks, the methodology is being developed and the tools are becoming available.

***Campylobacter* spp.** A commercially available, alkaline phosphatase-labeled oligonucleotide probe specific for thermotolerant campylobacters detected all 71 *C. jejuni* and *C. coli* strains tested.[68,115] A different commercial, non-isotopic DNA probe was tested on enrichments from contaminated chicken carcasses and from feces.[323] In the enrichment about 10^6 colony forming units (cfu) ml^{-1} were required to yield a positive result.

DNA extracted from pure cultures provided template DNA for a set of *flaA* (flagellar antigen gene) PCR primers.[266] Reported sensitivity was four cells per reaction but dropped to 30 to 60 cells per reaction in seeded stools. To detect campylobacters in raw milk and dairy products, Wegmüller et al.[366] digested samples adjusted with buffer and removed the fatty acid and water layers by centrifugation. After lysing cells with proteinase K, primers to *flaA* and *flaB* genes were used to amplify an intercistronic region predicted to be between 340 and 380 basepairs (bp) long. To ensure specificity, a triple primer PCR was used (the third primer was targeted to the intercistronic region) and a seminested PCR was carried out. The limit of detection in water was between 10 to 100 cells after 50 cycles of amplification using naturally contaminated samples, suggesting that PCR-inhibiting substances were present. A three-primer, seminested PCR could detect less than 10 cells in 40 ml of raw milk or 2 g of soft cheese after rounds of 30 and 40 cycles. Environmental water samples were collected onto Fluoropore, Durapore, and cellulose acetate filters and PCR was used to test for the *flaA* gene.[265] No PCR amplification was noted with the cellulose acetate filters but sensitivity with the other filters was about 10 to 100 viable cells per 100 ml water without enrichment.

By targeting various regions of *Campylobacter* 16S rRNA, a 426 bp amplicon was observed when *C. jejuni*, *C. coli*, and C. *lari* were tested.[123] For seeded

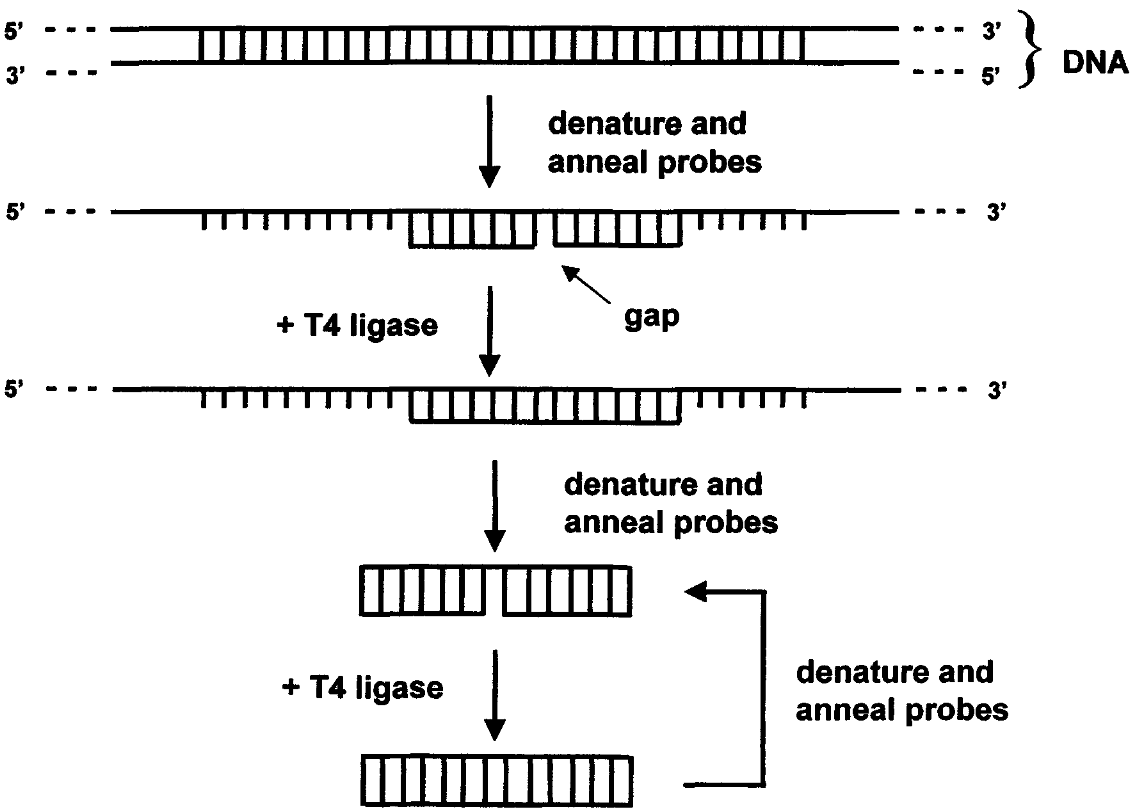
5'
3'
3'
5'
DNA
denature and anneal probes
5'
3'
gap
+ T4 ligase
5'
3'
denature and anneal probes
+ T4 ligase
denature and anneal probes

Figure 64–7 Ligase Amplification Reaction

chicken product samples, a detection limit of about 12 cfu per reaction (corresponding to 500 cfu ml^{-1} of enrichment medium) was reported and results were available within 48 hours. In a method employing PCR and specific DNA probes, Giesendorf et al.[124] targeted arbitrary sequences in conjunction with ERIC (enterobacterial repetitive intergenic consensus sequences[159]) to generate species-specific fragments for use as DNA probes.

The isothermal DNA amplification technique, NASBA™ targeted to 16S rRNA, was specific for *C. jejuni, C. coli,* and *C. lari.*[353,354] Sensitivity was about 1000 rRNA copies per reaction; when chicken skin and ground beef were seeded, 3 *C. jejuni* cells g^{-1} could be detected after enrichment, in a background of over 10^6 Gram-negative cells.

Clostridium botulinum. Because neurotoxins elaborated by *C. botulinum* (see Chapter 41) can cause severe and sometimes fatal paralytic disease, rapid detection and identification of the presence of these toxins in foods are critical. The effects of *C. botulinum* (with the exception of infant botulism) are due to the ingestion of preformed toxin. Therefore, viable cells or spores of *C. botulinum* might not be present in a food suspected of containing the toxin. Because PCR can be used to amplify the DNA of viable but nonculturable or even nonviable microbes, this method may be employed to demonstrate whether a food has been contaminated with *C. botulinum* harboring a particular toxin gene.

By targeting the 16S rRNA gene sequences, a PCR-based genus-specific method was developed with a claimed sensitivity for *Clostridium* spp. spiked into soft drinks of about 5 cells or spores per reaction.[118] Most PCR-based tests for *C. botulinum* detect neurotoxin gene sequences. Using the published amino acid sequence of the type B neurotoxin, Szabo et al.[327] selected primers but reduced their degeneracy by considering codon usage by other clostridial genes and the codon bias exhibited by the related tetanus toxin gene. A test sensitivity of 10 pg (about 2 x 10^3 cells) of *C. botulinum* DNA was achieved when PCR products were analyzed by gel electrophoresis, but using biotinylated PCR-product that was hybridized to gel blots the sensitivity was improved 100-fold.

The published nucleotide sequence of the *C. botulinum* type A neurotoxin gene[33,340] allowed primer selection for a 1.3 kilobase pair (kbp) region.[98,100] Commercially canned products were inoculated with 100 viable spores per gram. After incubation samples were centrifuged, pellets were washed in buffer and then heated at 99° C for 10 minutes to release the DNA from vegetative cells for amplification. Products were detected by dot blots by using a digoxigenin-labeled internal gene probe. Most food samples gave positive results after 1 day of incubation while others required more than 15 days. The different results obtained with some food products were probably due largely to differences in the extent to which they allowed germination of spores of *C. botulinum* and multiplication of the vegetative bacteria. The lysing procedure was probably not adequate to release DNA from nongerminated spores.

The nucleotide sequences of *C. botulinum* type B, C, D, E, and G toxin genes share about 50% sequence similarity with type A toxin and tetanus toxin genes.[371] Individual primer sets were used to detect genes encoding toxin types A, B, C, D, and E with a sensitivity of about 10 fg of isolated DNA representing about 3 cells.[328] Similarly, other primer sets for toxin gene types A, B, and E were tested.[111] A PCR-based test specific for the type F toxin gene also was developed.[101] Degenerate primers were used to amplify a 1.1 kbp region of the genes encoding toxins A, B, E, F, and G from *C. botulinum*, *C. barati*, and *C. butyricum*.[48] A degenerate primer pair was used to amplify a 260 bp region from genes that encode toxin types A, B, E, F, and G.[93] Sensitivity was 1 vegetative cell per reaction for types A and B and 10 cells for type E. In food samples inoculated with 10 vegetative cells of *C. botulinum* g^{-1}, a positive result was obtained after enrichment for 18 hours at 20° C.

Clostridium perfringens. By specifically targeting the 16S rDNA, Wang et al.[364] developed a PCR-based method specific for *C. perfringens* and capable of detecting two cells from pure culture. The PCR-based method was able to detect *C. perfringens* from enrichments of food samples initially inoculated with 20 cells per 100 g. The sequence of an enterotoxin gene from *C. perfringens* has been determined and synthetic probes developed and tested.[355] PCR-based specific amplification of the *C. perfringens* enterotoxin gene has also been carried out.[303] An enterotoxin probe labeled with digoxigenin during amplification was used in a colony hybridization assay to detect and enumerate *C. perfringens* in raw ground beef seeded with known numbers of vegetative bacteria.[12] Fewer than 10 cfu g^{-1} of the sample could be detected. Enterotoxic strains were identified by PCR-amplification of regions of the alpha-toxin and enterotoxin genes from a single colony on tryptose-sulfite-cycloserine (TSC) agar without egg yolk.[182] PCR-based detection of the enterotoxin A gene was compared with a colony hybridization method for testing irradiated and nonirradiated beef samples; the PCR assay yielded false-positive results,[13] probably because some cells unable to form colonies still contained PCR-amplifiable DNA.

Escherichia coli. Four categories of *E. coli* cause foodborne illness in humans (see Chapter 43): ETEC, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC). This classification is based on virulence mechanisms. Many of the genetic elements responsible for virulence have been used as targets for molecular detection and identification methods, and these methods are being refined continually to improve their sensitivity and specificity. Enrichment in tryptic soy broth for 5 hours at 42.5° C, followed by a multiplex PCR to simultaneously detect and differentiate the various pathogenic strains as well as total *E. coli* from artificially contaminated ground beef, gave a sensitivity of $<10^2$ to 10^3 cells per 10 g.[221]

- ***EPEC.*** The pathogenicity of EPEC is attributed to genes present on a 55 to 70 Mda EPEC adherence factor (EAF) plasmid. A gene probe for a conserved section of this plasmid has been developed[253] and PCR amplification of a 397 bp region was reported.[112] This region had little homology with known sequences except for a section of the IS630 sequence of *Shigella sonnei*. Gene probe and PCR methods also have been used to detect the chromosomal *eae* gene cluster necessary for attachment and effacing.[170–172]

- ***EIEC.*** The invasive ability of EIEC strains is attributed to a large 120 to 140 Mda plasmid encoding several genes for outer membrane polypeptides[89] and several gene probes have been developed.[37,267,383] Lampel et al.[206] used PCR amplification of a 760 bp region of the plasmidborne invasive gene. A number of PCR template preparation methods from foods have been examined. A minimum detection level of 10^2 cfu ml^{-1} without enrichment was reported when the template was prepared by proteinase K treatment of milk followed by boiling and ethanol precipitation.[186] Columns (Promega, Madison, WI) eliminated PCR inhibitors and gave a minimum detection level of 10^3 cells ml^{-1} of milk without culturing.[7]

- ***ETEC.*** The heat-labile enterotoxin (LT) is immunologically related to cholera toxin. The LT genes (*elt*) are plasmid-encoded and present in both human (LTH or LT1) and piglet (LTp or LT2) strains. The heat-stable enterotoxins (ST) are low molecular weight, nonantigenic, and are also plasmid-encoded. The STaI and STaII forms are usually of human or animal origin while the STb form is commonly from porcine sources.

Colony blot hybridization can detect single genes or combinations.[147,155,297,311] Combinations of oligonucleotide probes to individual genes or alleles that respond similarly under the same hybridization conditions can be used for the simultaneous detection of various genetic sequences.[155,311] Oligonucleotide probes labeled with either ^{32}P or digoxigenin had approximately equal sensitivity for colony blots from stool samples spiked with 10^6 to 10^8 cfu g^{-1}, provided colony-lift filters were proteinase K-treated prior to hybridization with the digoxigenin-labeled probe.[311] A fused gene probe has been used to detect ETEC harboring the genes for either LT or ST or both.[297] Unique sequences from *estA* and *estB* genes (for ST) were cloned into a plasmid, which was linearized and transcribed with RNA polymerase to incorporate radioactive or biotinylated ribonucleotides into a single, hybrid RNA probe.

PCR methods have also been developed to detect LT and/or ST genes of ETEC. Enrichment culture prior to PCR amplification yielded sensitivities as low as 3 cfu initially inoculated into 25 g of minced meat.[368] When PCR amplification was applied directly to food samples without enrichment, 1,000 cfu g^{-1} soft cheese were detected.[5] Some approaches to improve PCR assay sensitivity include immunomagnetic selection,[157] enrichment,[368] ion exchange chromatography,[261] and gene probes to detect PCR products.[49,157,261,359] Nested PCR distinguished between the ST1a and ST1b alleles.[157] The first PCR amplified a 147-bp region for either ST1a or ST1b while the second PCR used two different primer sets to amplify a 124-bp fragment to specifically detect ST1a or ST1b. Restriction fragment length polymorphisms (RFLP) are used as an alternative method to distinguish between different alleles of the LT1 and ST1 enterotoxin genes.[49]

- ***EHEC.*** The serogroup most commonly associated with EHEC illness to date in humans is O157:H7 (see Chapter 43); therefore, many EHEC tests are specific for this serogroup. The O157:H7 serogroup often is characterized by its inability to use sorbitol and the lack of a functional b-glucuronidase; however, recent reports have described b-glucuronidase-positive strains (see Chapter 43). A base substitution at position 92 in the *uidA* (b-glucuronidase) gene has been identified as specific for *E. coli* O157:H7 serotype, although this base substitution does not cause the lack of b-glucuronidase activity.[96] This distinction provides a target for genetically based tests specific for *E. coli* O157:H7.

A 3.4 kbp segment of a 60 MDa plasmid containing genetic elements involved in adherence is common to most members of the O157:H7 serogroup.[222] The EHEC *eae* gene encodes an invasin that may also contribute to virulence.[225] The *eaeA* gene of O157 EHEC was cloned and sequenced.[20,224,393] The first 2.2 kbp exhibits 97% sequence homology with the EPEC *eae* gene but the sequence similarity in 800 bp of the 3' end is only 59%. The EHEC *hlyA* and *hlyC* genes have sequences unique to EHEC strains[306–308] and a number of genetic probes and PCR methods for the detection, identification, and/or characterization of EHEC target these genetic elements.

Poly- and oligonucleotide probes have been developed to detect the *slt*, EHEC *eaeA*, *uidA*, and/or EHEC plasmid genes.[30,31,66,122,304,312,338,339,342,376,377,380] For example, Samadpour et al.[304] used shiga-like toxin (SLT) I and SLT II probes to test oyster and raw hamburger. A minimum initial inoculum of 1.3 cfu O157:H7 g^{-1} of food sample was detected after enrichment for 16 to 24 hours at 37° C in modified tryptic soy broth (mTSB) followed by streaking to mTSA and colony hybridization or dot-blot from the enrichment. Although dot blot analysis was faster (no time was needed for colony growth), it did not allow recovery of the specific isolate from the food sample. Probes were used to screen raw meat, poultry, and seafood samples, after cultural enrichment, for *sltI* and *sltII* genes;[90,377] both studies reported the detection of the *slt* genes in 17% of the food samples tested.

Because multiple genetic elements are involved in the pathogenicity of EHEC organisms, multiplex PCR analyses have often been used to simultaneously screen for several genetic elements.[114,154,278,329,380]

The ability of PCR to detect *slt* genes in DNA extracted from stool samples was compared with colony blot hybridization, assays for free SLT, and with cultural methods for EHEC detection.[282] A minimum detection level of 10^3 and 10^4 cfu g^{-1} was reported when the bacteria were separated by centrifugation and washed prior to DNA extraction and PCR analysis. To detect EHEC in stool samples by multiplex PCR, bacteria were lysed by heating and proteinase K, DNA was extracted with phenol-chloroform, precipitated with ethanol, and purified with silica particles.[42] After ground beef inoculated with EHEC was incubated for 6 to 24 hours in mTSB, DNA was extracted using proteinase K and Sarkosyl and precipitated with sodium acetate and ethanol to prepare a template for a multiplex PCR for detecting *slt* genes. A minimum detection level of 1 cfu g^{-1} in the original meat sample was reported.[119] To detect *E. coli* O157 seeded into several types of food, the foods were homogenized in a selective enrichment medium and incubated at 37° C for 6 hours, during which time the number of target cells increased 5×10^3-fold.[174] The bacteria were either separated by centrifugation, washed, and resuspended in distilled water, or concentrated by using immunomagnetic beads. A multiplex PCR to detect fragments of the *sltI* and *sltII* genes had a sensitivity of about one cell g^{-1} of the original food. The sensitivity and specificity of PCR analyses have also been improved by various hybridization formats used to detect PCR-amplified products.[21,42,164,207]

In some PCR analyses, the primers are not selected to achieve specificity for particular genetic regions and the specificity is determined after PCR amplification. A degenerate set of PCR primers was selected from conserved regions of the *sltI* and *sltII* genes;[184] internal oligonucleotide probes that shared only 30% sequence homology then were used to distinguish between amplified product from the *sltI* and *sltII* genes. RFLPs were used[292,293,350] to distinguish between *sltII* and an *sltII* variant in PCR-amplified products.

The use of a MAMA to detect *E. coli* O157:H7 was described earlier and shown in Figure 64–5. When multiplexed with primers for the *sltI* and *sltII* genes,[55] this PCR detected *slt* gene-containing O157 strains.

Listeria. Of the six species comprising the genus *Listeria*, only *L. monocytogenes* is considered an important foodborne pathogen for humans (see Chapter 44). A number of molecular detection and identification methods using several targets have been reported for both the *Listeria* genus and specifically for *L. monocytogenes*.

A cloned DNA fragment based on a putative hemolysin gene was used by Datta et al.[74] as a probe for colony hybridization to enumerate *L. monocytogenes* in naturally contaminated dairy products. An oligonucleotide also based on the sequence of this region was used to detect *L. monocytogenes* in artificially contaminated raw milk and soft cheeses.[73] The DNA fragment later was shown to be part of the sequence designated the *msp* gene. This gene encodes a major secreted polypeptide of approximately 60kDa,[107] which also is called *iap* (invasion-associated protein).[204] The *msp* gene is conserved among *L. monocytogenes* strains, and probes for the *msp* gene, when used under stringent conditions, are specific for *L. monocytogenes*.[201] The fragment used by Datta et al.[73] was labeled with digoxigenin by the random primer technique and used in colony hybridization.[190]

Gene probes for *L. monocytogenes* based on the listeriolysin O gene also have been developed;[75,220,241] highly stringent hybridization conditions are required for specificity. An oligonucleotide probe[75] was applied to colony blot hybridization from ground beef samples.[245] Probe efficiency was dependent on the ability of the plating media to suppress background growth and the stringency of hybridization conditions. An oligonucleotide probe labeled with horseradish peroxidase was less sensitive than the radioactive labeled probe.[72] Herman and DeRidder[145] compared oligonucleotide probes for listeriolysin O and *iap* genes to detect *L. monocytogenes* in soft cheese; these probes were labeled either directly by coupling to horseradish peroxidase or by incorporation of digoxigenin but both produced background signals. Partial success with the digoxigenin system was reported after excess cellular debris was removed from the colony blots by rubbing with a soft paper soaked in 5XSSC solution.

The first commercially available gene probe product (GeneTrak) for *Listeria* spp. used a ^{32}P labeled synthetic probe.[195] The Gene-Trak Isotopic Assay and the US Food and Drug Administration (FDA) cultural method gave comparable results with spiked food samples but the Gene-Trak method was less sensitive when applied to naturally contaminated seafood samples.[238] A colorimetric version of the Gene-Trak gene probe hybridization is now available.[192] A 5.9% false-negative rate for the Gene-Trak system was reported with 250 naturally contaminated cheese and environmental samples, compared with the International Dairy Federation cultural method.[351] This may have been because the numbers of *Listeria* from samples may not have reached the minimum detection limit for the hybridization (10^6 cells ml^{-1}) during the cultural enrichment step.

Using a second commercially available kit (AccuProbe *L. monocytogenes*), the minimum detection level for bacteria resuspended in physiological saline is 10^5 cfu in 50 µl. A minimum of 10^6 cfu per 50 µl was required for samples from three different nonselective enrichment broths and the FDA *Listeria* enrichment broth. The broths UVM-I, UVM-II, and PALCAMY interfered with the hybridization.[257] Tests of 44 *Listeria* isolates resulted in the correct identification of all *L. monocytogenes* isolates and perfect correlation between a slot-blot hybridization using the probe developed by Datta et al.[72] and the AccuProbe *Listeria monocytogenes* kit.[176]

A region of the spacer between the 16S and 23S rRNA genes was specific for *Listeria* species with a minimum detection level of 5×10^2 cells ml^{-1} in artificially contaminated meat homogenates without enrichment.[92] Hybrids between *Listeria* rRNA and a biotinylated rDNA probe in an aqueous environment were captured by monoclonal antibodies to heteroduplex DNA.

PCR analyses have been developed to detect one or more genes specific either for *L. monocytogenes* or for all *Listeria* spp. The most common target is the *hylA* gene encoding listeriolysin O.[17,29,35,36,79,104,109,128,144,220] This target also has been used in multiplex PCR analyses.[39,51,65,99,116,178,210] Other virulence-related targets specific for *L. monocytogenes* include the *iap* gene,[44,51,99,116] the Dth-18 gene,[220,369] *prfA* and *plcB*,[65] and hemolysin genes.[116,220] Other genes specific for *L. monocytogenes* but not necessarily virulence-associated have been used.[57,176,220,287] The 16S rRNA gene is another PCR target either for detection of all *Listeria* spp.[39,210] or specifically for *L. monocytogenes*.[208,365]

Test sensitivity can be improved when applying PCR methods to foods for the detection of *Listeria* by a variety of template preparation protocols.[65,109,144,208,234,369]

PCR sensitivity was improved by the use of short denature and anneal times with a rapid transition and an increased number of cycles enabling detection of 4 to 40 cfu g^{-1} inoculated into diluted food samples that were not enriched.[365]

Other methods have been developed to improve PCR sensitivity. These include nested PCR,[167] internal DNA gene probes,[29,167] RNA probes,[35,36] and the degradation of an internal probe during the amplification process (see Section in 64.5).[17] The ligase chain reaction discriminates between *L. monocytogenes* and other *Listeria* spp. based on a single basepair difference in the V9 region of the 16S rRNA gene.[374]

Salmonella**.** Methods have been designed to detect the genus *Salmonella* because most species are associated with illness in humans. One of the first hybridization tests used a ^{32}P-labeled probe for chromosomally located *Salmonella*-specific sequences.[105] This probe was used in the commercially available kit (GeneTrak) and gave comparable results to standard culture methods when tested on more than 1,600 samples representing 23 food types.[108] This kit also was used to detect the DNA of *Salmonella* spp. extracted directly from filter-concentrated water samples.[196]

A second GeneTrak colorimetric kit targeted the *Salmonella* rRNA genes, which are present in multiple copies. Comparisons were made between this kit and conventional cultural and other rapid method tests based on enzyme linked-immunoassay (EIA) techniques.[69,70,286,381] D'Aoust et al.[70] also explored the use of alternative cultural conditions to improve the performance of the Gene-Trak method. Components in some selective enrichment broths appear to inhibit the gene probe assay. The probing of a combination of 6-hour Gram-negative broth postenrichment cultures inoculated from 24-hour tetrathionate brilliant green and selenite cystine enrichment enhanced the Gene-Trak assay sensitivity and specificity.

Other probes have been used for detection of *Salmonella* spp.[262,280,309,348] Wicking can separate DNA from substances that compromise DNA retention and hybridization.[309] Cells are lysed rapidly and DNA denatured using an alkali-surfactant reagent. Then, the vertical capillary action of Hybriwix filters allows DNA to migrate up the filter.

Biotin was used as the reporter molecule in a DNA probe/membrane method;[348] both dot blots from preenrichments and selective enrichments and colony blots were used. Detection of 1.6 *Salmonella* cells g^{-1} food was reported. To reduce nonspecific background, colonies should be small to allow for complete hydrolysis by proteinase K.

Salmonella virulence genes also have been used as targets. Probes to the *invA-D* genes needed for invasion of intestinal epithelial cells were used,[117] and a synthetic oligonucleotide incorporating a 1 bp difference in the plasmidborne virulence gene (*spvA*) produced a gene probe specific for *S. enteritidis*.[140]

PCR-based assays for *Salmonella* detection have used the following targets: the origin of *Salmonella* chromosome DNA replication (*oriC*),[110,373] the structural gene for thin aggregative fimbrae (*agfA*),[85] a gene for DNA binding proteins (*himA*,[23] *hns*[180]), genes for invasin proteins (*invA*, *invE*[324,325]), tyvelose-precursor synthetase—one of the genes involved in the biosynthesis of the *Salmonella* O antigens (*rfbS*),[227] *phoP*,[274] *phoE*,[198] insertion sequence 200 (IS200),[52] and other *Salmonella*-specific genetic sequences.[1,321]

Several template preparation approaches have been used for PCR-based detection. The efficiency of six different DNA extraction protocols was determined after a short cultural enrichment.[321] The most reliable, rapid, and sensitive results were obtained using Proteinase K and/or a centrifugation step to concentrate the samples. Others used guanidinium isothiocyanate following a short enrichment step,[23] or guanidinium isothiocyanate and Chelex[180] to extract DNA from oyster samples at PCR detection levels from 1–10 cells to <40 cells g^{-1} homogenized oysters. Chelex also has been used to prepare PCR template DNA from other food and environmental samples.[52,198] IMS using magnetic beads coated with six monoclonal antibodies specific for *Salmonella* serogroups A–E was an efficient way to remove and concentrate target *Salmonella* from samples such as chicken meat[110] or feces.[373]

Methods to detect PCR-amplified products from *Salmonella* include digoxigenin-labeled probes to internal sequences of the amplified products[110] and hybridization formats to "capture" amplified sequences with oligonucleotides covalently bound to microtiter wells.[52] Some applications incorporate biotin into primers[320] and both digoxigenin labeled dNTPs and biotin labeled primers.[227] Chromogenic substrates of peroxidase and alkaline phosphatase for colorimetric tests and Lumi-Phos 530 chemiluminescent substrates were compared.[320] The minimum detection levels of target DNA per microtiter plate well for chemiluminescence, peroxidase, and alkaline phosphatase methods were 1.76 fmol, 4–5 fmol, and 8–10 fmol, respectively, whereas that for agarose gel analysis was 8.8 fmol. When pure cultures were tested, initial levels of 5 cells and 50 cells could be detected by chemiluminescence and colorimetry, respectively, after amplification.

Shigella. The genus *Shigella* contains four spp: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* (see Chapter 46). Genetic techniques used to detect *Shigella* spp. target both plasmid and chromosomally located virulence-associated genes. All *Shigella* spp. show greater than 80% DNA sequence similarity to *E. coli*. *Shigella* and EIEC possess similar plasmids required for virulence and this has resulted in probe and PCR methods capable of detecting both *Shigella* and some enterovirulent *E. coli* groups.

The earliest reported DNA hybridization methods used ^{32}P labeled probes to the virulence plasmid detected in *Shigella* spp. and EIEC.[37,319] Two probes were compared with the Serèny invasiveness test to characterize 42 *Shigella* and 29 *E. coli* isolates.[383] Three Serèny-test-negative isolates hybridized with both probes, suggesting that additional factors may be required for virulence. When the Boileau et al.[37] probe was applied to the direct detection of *Shigella* and EIEC in stool blots incubated overnight on MacConkey agar, the specificity for *Shigella* and EIEC remained high, but the sensitivity was poor compared with that of other cultural methods. This was attributed in part to inhibition of *Shigella* and EIEC by other fecal bacteria.[334] An alkaline phosphatase-conjugated oligonucleotide DNA probe selected from a 2.5 kb probe[319] was compared with the Serèny test by using a dot-blot format for the testing of both *Shigella* and EIEC.[269] The probe hybridized with all 52 Serèny-test-positive isolates and 4 of 21 Serèny-test-negative isolates.

Invasion plasmid antigen (*ipa*) gene probes also specifically detect *Shigella* and EIEC.[259,357,358] The *ipaH* gene, a multiple-copy element encoding a 60 kdal antigen, is located both chromosomally and on the invasion plasmid; it was selected as a target and the probe was labeled with ^{32}P[357] and suberyl-alkaline phosphatase.[259]

Other genetic elements in *Shigella* used as PCR targets include *ial* genes[90,113,161,206,390] and *ipaH* genes[162,313,314] for detection of *Shigella* spp. and EIEC. The Shiga-toxin gene and the related Shiga-like toxin gene are often used to detect *Shigella* spp. and EHEC.[164,277,278,337]

Magnetic beads coupled to monoclonal antibodies specifically captured *S. dysenteriae* and *S. flexneri* from feces, without enrichment, to prepare a PCR template for amplification of an *ial* gene fragment.[161] An alkaline phosphatase-conjugated probe was used to detect the amplified product; a minimum detection level of 10 cfu g^{-1} was achieved. When lettuce samples were tested using enrichment but without use of magnetic beads, 10^4 *S. flexneri* g^{-1} were detected.[206] The enrichment was kept short to reduce plasmid loss.

Staphylococcus aureus. *S. aureus* causes disease by formation of toxins in food (see Chapter 47). Identification of toxin type is important for epidemiological purposes.

Relying on published sequences, a set of primers was selected to detect the five types of staphylococcal enterotoxin genes and a procedure involving lysozyme, lysostaphin, and sarkosyl was used to lyse cells.[177] Using pure cultures, a sensitivity of roughly

100 toxin-producing cells was achieved when PCR products were detected by gel electrophoresis. If cells were lysed by heat treatment, positive results required at least 10^5 cells, illustrating the relative difficulty of extracting DNA from Gram-positive cells. Gene probes specific for the individual toxin types have also been developed.[168]

Staphylococci that elaborate enterotoxin type C may contaminate milk as a result of bovine mastitis. The structural genes encoding staphylococcal enterotoxins B (*entB*) and C (*entC1*) have been amplified,[378] and nested primers were used to PCR-amplify an internal segment. Primers to amplify the *entB* gene encoding type B staphylococcal enterotoxin enabled detection of about 10^5 cfu ml^{-1} of *S. aureus* seeded into milk. This sensitivity appears to be an order of magnitude or two less than in other reports,[378] suggesting PCR-inhibiting substances may have been present. With nested primers, about 1 fg per reaction of purified DNA (approximately 10 cells) could be detected. When a single set of primers was used, sensitivity was about 100 pg per reaction. Using PCR primers specific for genes for toxin types A, D, or E, detection sensitivity was about 1 cell per reaction for pure cultures and 1–10 cells g^{-1} in seeded foods after enrichment.[347] An *S. aureus*-specific gene probe to a unique region of the 16S rRNA gene was reported.[27] A nonisotopic, digoxigenin-labeled probe based on total genomic DNA was used to specifically detect *S. aureus* in meat and milk even though other staphylococci were present.[379]

***Vibrio* spp.** *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are the most important disease-causing members of this genus (see Chapter 48). Much nucleotide sequence information is available for virulence factors of these species. Ribotyping has also been used in epidemiology.[360] Various regions of the 16S rRNA gene are target sites for both genus-specific and species-specific gene probes and PCR primers.[87] Coelho et al.[63] constructed specific synthetic oligonucleotides for the detection of Vibrios.

The genes encoding the cholera enterotoxin (*ctxA* and *ctxB*), a major virulence factor of *V. cholerae* O1, are the targets of gene probe and PCR-based tests,[45,102,136,197,281,298,318,356,385,391] despite showing sequence similarity with the heat-labile enterotoxin gene of *E. coli* and a gene in *Salmonella*.[248,249] An oligonucleotide probe specific for a heat-stable enterotoxin produced by some strains of *V. cholerae* non-O1 (NAG-ST) showed that some *V. cholerae* O1 strains also contained this gene.[331]

Oysters, crabmeat, shrimp, and lettuce were seeded with *V. cholerae* El Tor Inaba from an ill patient, and the samples were enriched.[200] After incubation, PCR templates were prepared by boiling cultures and regions of the A and B subunit genes were amplified. Initial levels of *V. cholerae* of 10 cfu g^{-1} in oysters and 1 cfu g^{-1} in lettuce could be detected after 6 hours of enrichment.

The cytolysin gene of *V. vulnificus* has been sequenced[388] and primers selected.[152] To overcome inhibition of amplification by oyster meat, template preparation methods were evaluated. After 24 hours in enrichment medium cells were disrupted by guanidine thiocyanate and extracted with chloroform; an initial level of 10^2 cells g^{-1} oyster meat was detected. A fluorescently labeled eubacterial oligonucleotide probe to 16S rRNA genes was used to detect and enumerate *V. vulnificus* in liquid or colony hybridization formats.[142] A nonisotopic, *V. vulnificus*-specific probe to the cytolysin (*hylA*) gene could detect about 10^2 cells ml^{-1} of seawater.[386]

The majority of *V. parahaemolyticus* strains recovered from the environment lack hemolysin genes associated with virulence.[244,317] Thus, screening isolates for the hemolysin gene(s) may enable the identification of pathogenic strains; however, a family of related hemolysins complicate PCR analysis.[11,193,258,329,330] A probe for the thermostable direct hemolysin (*tdh*) gene was specific for *V. parahaemolyticus*.[213] Enzyme-labeled probes for the *tdh* gene and the TDH-related hemolysin gene (*trh*) were developed, but the *tdh* probe hybridized with some strains of *V. hollisae* and *V. mimicus*.[387] Lee and Pan [214] PCR-amplified the *tdh* gene with a sensitivity of 10^3 cells from a broth culture. A target of unknown function but specific for *V. parahaemolyticus* allowed detection, after enrichment, of 9.3 cfu g^{-1} of seeded oysters.[215]

There may be situations when all three species of *Vibrio* are sought in the same sample. Trost et al.[344] chose primer sets for the enterotoxin, the thermostable direct hemolysin, and the cytolysin genes of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, respectively. These primers functioned under the same PCR conditions and generated PCR products of different sizes that could be distinguished by gel electrophoresis.

***Yersinia enterocolitica* and *Yersinia pseudotuberculosis*.** Many serotypes of *Yersinia enterocolitica* exist in nature but only a few are associated with diseases in humans (see Chapter 49). A family of 60 to 70 kbp plasmids, widely distributed in the yersiniae, is essential for pathogenicity but can be lost during growth or prolonged storage. Several chromosomal genes are required for virulence including *inv* and *ail* encoding factors for the invasion of eukaryotic cells[243] and *yst*-encoding a *Y. enterocolitica* heat-stable enterotoxin.[273]

Certain serovars of *Y. enterocolitica* are more prevalent in Europe while other groups are found more frequently in North America, although this situation is changing.[216] A set of *yst* primers differentiated between two groups of *Y. enterocolitica.*[160] Serogroup O:3 isolates recovered in Europe were characterized by a single 200-bp PCR product that matched the size predicted by the nucleotide sequence;[78] however, when serogroup O:8 strains common in North America were tested, a 1.6 kbp fragment and a 1.4 kbp fragment were observed. Such discrimination can be of epidemiological use.

A PCR-amplification of a fragment of the *ail* (attachment invasion locus) gene indicated that all 48 porcine strains and 5 clinical isolates were positive. Gene probes for *ail* in *Y. enterocolitica* and *inv* in *Y. pseudotuberculosis* are species specific.[95] Using an *inv* gene target for *Y. pseudotuberculosis*, the *ail* gene for *Y. enterocolitica*, and the *virF* primer set of Wren and Tabaqchali[384] specific for virulence plasmids found in strains of both species, Nakajima et al.[252] used PCR to differentiate between pathogenic and nonpathogenic strains. Targeting *yadA*, six pathogenic serogroups of *Y. enterocolitica* were identified with a sensitivity of 10 to 30 cfu g^{-1} of meat in the presence of a 10^6-fold excess of background microorganisms.[183] Overnight enrichment in non-selective medium increased sensitivity to about 2 cfu g^{-1} unless the number of background microorganisms was greater than 10^7 cfu g^{-1}.

Because *Y. enterocolitica* was found in the blood of asymptomatic donors, Feng et al.[97] used both a chromosomal target (*ail*) and a plasmid target (*virF*) to detect virulent strains directly in blood by using PCR. Sensitivity was 500 cfu per 100 ml of blood, but no enrichment or other method was used that would avoid possible amplification inhibitors and increase the concentration of bacteria.

It is not always clear whether plasmid or chromosomal genes should be chosen as targets; a disadvantage of the former is that loss of virulence plasmids can occur during laboratory culture.[149,151,395] In some cases both plasmid and chromosomally encoded virulence genes are required to produce disease; lack of either gene renders the cell avirulent. Because multiple primer sets can be used within the same PCR tube (multiplex PCR), it would be a reasonable strategy to test for the presence of both genes. The presence of either gene alone might indicate pathogenic potential but only strains harboring both chromosomal- and plasmid-borne genes would be considered pathogenic.

64.7.2 Viruses

Gene probes and PCR are being used to test for foodborne viruses and can detect fewer than 10 virus particles. RNA-genome viruses can be detected by the use of RT to form cDNA that serves as a PCR template.

Norwalk Virus. This virus is probably the leading cause of non-bacterial gastroenteritis among adults in the US (see Chapter 52) and cannot be grown in cell culture. RT coupled PCR (RT-PCR) can detect Norwalk virus in stools[173] and in shellfish.[394] Extraction of viral RNA from oysters by homogenization, organic flocculation, and polyethylene glycol[9] resulted in a PCR sensitivity of about 50 to 500 seeded Norwalk virus particles per gram of oyster compared with 1.5 to 15 particles per reaction from seeded stool samples. After Norwalk virus was seeded into shellfish, genomes were recovered by treatment with guanidinium isothiocyanate and hydroxyapatite followed by cetyltrimethylammonium bromide and ethanol precipitation.[131] Primers targeted to the RNA polymerase gene allowed detection of 20 to 200 molecules of Norwalk virus RNA per reaction. Similar results were obtained for Norwalk-like viruses (small round-structured viruses, SRSV) seeded into oysters and mussels as well as in shellfish implicated in human gastroenteritis.[218]

An outbreak on a cruise ship allowed the comparison of PCR detection and identification with serological techniques.[189] Of 19 fecal specimens examined, 7, 19, and 5 were positive by electron microscopy, PCR, and immunoassay, respectively. The PCR-amplified region shared only 81% homology with that of a reference strain, suggesting an explanation for the low detection rate by enzyme-linked immunosorbent assay (ELISA).

Fresh-cut fruit was implicated as the carrier of Norwalk virus in an outbreak on a cruise ship. Of 10 fecal specimens assayed, 4 were positive by RT-PCR and the nucleotide sequence of the amplified product helped to identify the viral strain.[146] Consumption of contaminated oysters was suspected in a multistate outbreak; 20 of 26 (77%) samples were positive by RT-PCR.[88] Antigenically distinct SRSVs have been differentiated by using RT-PCR, taking advantage of nucleotide sequence differences.[8] Fecal samples from 19 outbreaks were examined by RT-PCR; 64%, 40%, and 8% of the samples were positive from outbreaks involving Norwalk virus, Norwalk-related viruses, or SRSV, respectively.[8]

To increase the sensitivity of PCR for Norwalk virus detection, De Leon et al.[77] used an RT-PCR-OP (oligoprobe). After removing RT-PCR inhibitors in fecal specimens by Sephadex G-200 gel chromatography, two sets of primers amplified regions of the RNA polymerase gene and a putative antigenic protein. Amplification products were detected with a digoxigenin-labeled oligonucleotide. A stool sample contained about 10^6 virions ml^{-1} as determined by immunoelectron microscopy and after a 10^4-fold dilution 10 μl still gave a positive amplification; therefore, the sensitivity of this method was close to one virion.

Rotavirus. Serotype-specific cDNA molecules were generated as gene probes to detect rotaviruses of porcine origin.[287] Gouvea et al.[130] targeted the major outer capsid glycoprotein gene (vp7) to detect rotavirus in fecal samples from humans with a sensitivity of about 2 ng (equivalent to 10^8 viral genomes). A PCR typing method was developed where each serotype resulted in a fragment of a characteristic size. Purification of templates by binding rotaviral RNA to a CF11 cellulose column in buffered ethanol increased sensitivity more than 1000-fold, presumably through the removal of PCR inhibitors.[375]

***Hepatitis A Virus* (HAV).** A number of seafoods (such as oysters) are consumed raw and, if grown in contaminated waters, can spread HAV to humans;[81] HAV can survive for up to several months in experimentally contaminated environments. HAV is easily transmitted by the fecal-oral route, and the period of communicability precedes the onset of clinical symptoms; thus rapid detection is essential for containing the spread of disease.

Jansen et al.[166] selectively captured virus particles in fecal samples by using anti-HAV monoclonal antibodies, released viral RNA by heating, and amplified it by RT-PCR. Sensitivity was 3 to 30 virus particles per reaction; Southern blot analysis of PCR products yielded a detection rate of 90%, whereas solid-phase radioimmunoassay and cDNA-RNA hybridization resulted in 50% and 65% positive samples, respectively. Use of antigen capture and RT-PCR to test seeded oysters and detection of PCR products with gene probes gave a sensitivity of about 4 virus particles per reaction.[80] Atmar et al.[9] attempted to detect HAV in seeded oysters by using a sample preparation method developed for Norwalk virus. Using a RT-PCR method, HAV could not be detected in seeded oysters but was detected if the virus was added to extracts of oysters. It was found that HAV was removed from the supernatant during the flocculation step. This finding illustrates the difficulty in extrapolating methods from one microbe and sample type to others. When RNA isolated from clams was used in RT-PCR,[129] 10 viral RNA molecules were detected in seeded samples. By using riboprobes, 67% of shellfish examined were found to contain HAV RNA.[219] There was no correlation between viral contamination and fecal coliform counts.

Enteroviruses. These RNA-containing viruses are a diverse group of pathogens and a common cause of childhood infections, the most notable of which is polio (see Chapter 52). It is difficult to detect these viruses with gene probes because titers of virus in clinical samples are usually low. Because the complete nucleotide sequences for several enteroviruses have been determined, RT-PCR methods have been applied.[288] A conserved region at the 5' end of the enteroviral genome was targeted. Assay sensitivity was in the range of 10^6 molecules per reaction; more importantly, the time required to detect and identify these viruses was shortened from 4 to 8 days by tissue culture to less than 1 day.

Using poliovirus seeded into oysters as a test system, Atmar et al.[9] found that flocculation and polyethylene glycol precipitation left RT-PCR inhibitors in the extracts but that they could be removed by CTAB (cetyltrimethylammonium bromide) allowing detection of 10 plaque-forming units. Similar results were reported by Lees et al.[217] Positively charged nylon membranes were used to capture enteroviruses from water samples, and RNA was isolated with oligonucleotides bound to magnetic beads.[125] The detection limit was 20 times the amount required to infect 50% of tissue culture cells (equivalent to about 1,000 virions in an original volume of 1 l). Of 40 water samples tested, 7 (17%) were positive.

64.7.3 Parasites

Cryptosporidium. Target genes for PCR include the 18S rRNA gene,[10,175] the 5' end of 18S and 5.8S rRNA genes,[47] and other *Cryptosporidium*-specific genes.[61,103,211] The sensitivity of PCR analyses was reduced up to 1000-fold when water samples were used instead of pure cultures. The use of flow cytometry or IMS separated *Cryptosporidium* from PCR-inhibiting substances.[175] Detection of PCR-amplified products with chemiluminescently labeled oligonucleotide probes also improved the PCR sensitivity 10 to 1000-fold.[175]

Entamoeba. DNA probes to rRNA can detect *E. histolytica;*[305] other probes distinguish between pathogenic and nonpathogenic isolates.[120,333] A PCR assay amplifies genetic sequences specific for *E. histolytica.*[332] Unique RFLP patterns for PCR-amplified product allowed pathogenic and nonpathogenic isolates to be distinguished.[332]

Giardia. *Giardia duodenalis* is the most frequently identified cause of waterborne intestinal disease in the US; *G. lambia* also is isolated from humans. Disruption of *Giardia* cysts with glass beads and Proteinase K was the most efficient protocol for extracting nucleic acids prior to DNA probe hybridization specific for the small rRNA subunit.[2] The giardin gene of *Giardia* has been identified as a target for the selection of PCR primers and internal probes for the detection either of *Giardia* cysts or to discriminate between *Giardia duodenalis* and other *Giardia* spp.[231,232]

64.8 TYPING OF FOODBORNE MICROBES

64.8.1 Introduction

DNA subtyping can be used in studies of the epidemiology of foodborne disease. In many reports, the analysis of DNA subtyping patterns of bacteria has been limited to visual inspection and manual classification into groups; however, similarity values between nucleotide sequences or gel patterns from isolates can be used to generate clusters. Dendrograms (ie, tree diagrams) can be used to visualize and sometimes to quantify the relationships between strains of a given bacterial species. Other pattern analysis and visualization techniques, such as principal coordinate analysis (PCoA), can be used to explore groupings of subtyping data. By using these methods, a new isolate can be compared with a database of previously classified isolates to designate the most similar ones. Each phenotype-based subtyping method such as serotyping, phage typing, and biotyping has its associated advantages and disadvantages, such as the number of groups generated and the problem of untypeable strains. DNA subtyping methods, although sometimes labor-intensive, have some advantages, and generally use commercially available reagents, rather than specialized materials such as specific antisera or collections of typing phages.

In general, DNA subtyping methods fragment genomic DNA to generate a pattern of fragment sizes or a nucleotide sequence (which is also a pattern). The most commonly used subtyping methods for characterizing bacterial genomes are PFGE,[34] ribotyping,[32] random amplified polymorphic DNA (RAPD),[367] or DNA sequencing.[264]

64.8.2 Restriction Fragment Length Polymorphism (RFLP)-Based Methods

Pulsed-Field Gel Electrophoresis (PFGE). Restriction enzymes that cut DNA infrequently are used to generate a low number (about 20 to 30) of fragments.[236] Most of these fragments are quite large (approximately 10 to 1,000 kbp) and need special electrophoretic separation methods.[34] The resulting fragment patterns represent the position of cleavage sites for a particular restriction enzyme and are used to group isolates.[127] Guidelines for bacterial subtyping by using PFGE DNA restriction fragment patterns have been published.[336]

Ribotyping. This reveals the distance between restriction enzyme sites on DNA fragments encoding genes for ribosomal RNA molecules. The restriction enzymes cleave DNA molecules to produce between about 500 and 1,000 fragments with an average size of about 1 to 20 kbp, which are separated by gel electrophoresis. Because this number of bands cannot be resolved readily, bands containing ribosomal sequences only are detected by hybridization with a probe encoding *E. coli* ribosomal RNA genes. These simplified patterns are used to discriminate between strains.[133,199]

PCR/RFLP. Restriction enzyme analysis of PCR-amplified DNA can be a rapid and powerful tool for discriminating between strains. For example, the 16S-23S rDNA spacer regions in *Staphylococcus aureus* were analyzed in this way.[137] Clear differences were observed between *C. perfringens* and *C. botulinum* and between *C. botulinum* neurotoxin types C and G.[138]

64.8.3 Random Amplified Polymorphic DNA

In the standard PCR, specific oligonucleotide primers are used to generate a segment that usually does not vary between isolates. In contrast the DNA produced by the RAPD method results in patterns that can be used to compare strains. These patterns arise from using a single, short primer (about 10 nucleotides) with reaction conditions that allow mispairing of primer and template DNA; thus, the primer will anneal at many different places throughout the genome. Randomly, some primers will be

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positioned such that amplification can occur in conjunction with other nearby primers. A number of regions will be amplified that are bounded by nucleotide sequences showing similarity to the primers. Because bacteria have different nucleotide sequences, they may exhibit distinguishable patterns. Although this technique appears to be difficult to conduct reproducibly (probably because of variations in template concentration and thermocycling), it is of interest because of its relative simplicity and speed.[126,271] RAPD analysis has been used to discriminate between strains of *E. coli*,[54] *Y. enterocolitica*,[283] *V. vulnificus*,[264] and *Salmonella enteritidis*.[299] In some cases, RAPD can distinguish strains that appear identical by multilocus enzyme electrophoresis.[363]

64.8.4 DNA Sequencing

Perhaps the eventual limit on the description of an organism will be the nucleotide sequence of its genome. Without requiring these data for the entire genome, it is possible to make taxonomic assignments by sequencing some regions of the genome that are variable between species but conserved within species, eg, the genes for the ribosomal RNAs. Nucleotide sequences of 16S rRNA genes from several species of foodborne pathogenic bacteria were retrieved from the GenBank® database[82] or the Ribosomal Database Project,[209] and aligned to generate a similarity matrix. By using hierarchical cluster analysis,[242] these sequences were grouped according to their similarity, which closely reflected their taxonomic position.[153] Gel banding patterns can be treated in a similar fashion but additional care must be taken to consider the variation in band peak position.[275]

PCR can be used in conjunction with sequencing to analyze bacterial colonies. One PCR adaptation employs unequal concentrations of primers so that one product strand will predominate and be available for sequencing.[139]

64.8.5 Examples Using *Vibrio cholerae*

Prompted by an epidemic in Latin America, the body of evidence on the molecular genetic epidemiology of *V. cholerae* based on several techniques is increasing rapidly. Olsvik et al.[264] developed a streamlined sequencing protocol based on PCR-amplified *ctxB* DNA to assist the rapid collection of this type of subtyping data. Based on the RFLP analysis of rRNA and *ctxA* genes, DNA sequences of the *ctxB* gene, PFGE, and multiple locus enzyme electrophoresis (MEE), Wachsmuth et al.[361,362] concluded that the Latin American clone of *V. cholerae* is closely related to the seventh pandemic strain. Similarly, the nucleotide sequence of the *ctxB* gene of *V. cholerae* O139, the strain responsible for outbreaks throughout Southeast Asia, resembles that of the classical biotype more than that of El Tor strains.[212] The RAPD fingerprinting method was applied to O139 strains, and the resulting patterns indicate that they comprise a unique subgroup genetically distinct from O1 strains and possibly arising from the El Tor biotype.[28,233] Similar DNA subtyping data were obtained using PCR, ribotyping, and PFGE on recently isolated strains from patients, food, and the environment.[352]

64.9 CONCLUDING REMARKS

The use of gene probes and the PCR has increased dramatically within the past few years, and a considerable number of variations on these basic techniques have been devised. Although most of the applications of these methods have been for clinical and environmental samples, the use of these methods to detect pathogenic microorganisms in foods is increasing. Virtually every important foodborne pathogenic bacterial species has been studied and gene probes and PCR primer sets developed for their detection and identification. Both virulence genes, to detect pathogenic strains, and rRNA genes, to detect particular taxonomic groups, are being used as probes and/or PCR targets. A considerable amount of effort is being expended to develop efficient and rapid protocols for the preparation of target DNAs from complex food matrices and, in some cases, considerable progress has been made. These methods, along with ribotyping, PFGE, and random amplified polymorphic DNA, also have been used to subtype strains and generate important epidemiological information for analyzing outbreaks of foodborne disease.

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65 Risk and Microbiological Criteria

Tony C. Baird-Parker and R. Bruce Tompkin

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65.1 INTRODUCTION

Since microorganisms were first recognized by man to cause foodborne illness and to spoil foods, microbiologists have been concerned with assessing the microbiological risks associated with foodstuffs and identifying means for their reduction, management, and control.

In the early part of the 20th century, safety concerns led to the development of performance criteria for thermally processed foods based on an estimation of the microbial kill needed to produce a safe food. The best known examples of these are the "Botulinum Cook," a thermal process designed to reduce the probability of survival of spores of *Clostridium botulinum* by a factor of 10^{12} and pasteurization, introduced to reduce the numbers of infectious pathogenic microorganisms present in raw milk to safe levels (see Chapter 3).

Similar concerns over salmonellas and other infectious and toxigenic microorganisms in raw and processed foods led to the development of microbiological criteria. These are used to assess whether an amount of food contains a concentration of a microorganism or a microbial toxin in excess of that regarded as acceptable. The first of such criteria had little scientific basis and Sedwick, referring to the basis of many microbiological standards, stated, "Standards are often the quote of one worker, easily seized upon, and quoted and requoted until they assume a semblance of authority."[64]

It was not until the early 1970s that microbiological criteria were placed on a sound basis by the International Commission on Microbiological Specifications for Foods (ICMSF).[35] The ICMSF introduced the use of statistically based sampling plans as part of a microbiological criterion and based the stringency of such plans on a consideration of three elements of hazard and risk. First, the degree of severity and extent of illness potentially caused by a microorganism or toxin contaminating a food. Second, the relative sensitivity of the intended consumer of the food to infection or intoxication by the food. Third, the likely fate of the microorganism (or toxin) between when the food is sampled and when it is consumed (ie, would it survive, increase, or be destroyed during any subsequent manufacturing process, during distribution, and/or preparation for use?). Based on these factors, the ICMSF proposed a matrix of 15 sampling plans (Table 65–1).[35] This matrix is used throughout the world by regulatory authorities and by industry for the establishment of microbiological criteria for foods.

Methods of microbiological risk assessment (MRA) and hazard analysis are of increasing interest to governments and the food industry. There are a number of reasons for this, including the potential cost/benefits to regulatory authorities of using risk assessments to prioritize public health concerns and allocating health resources accordingly,[1] the use by industry of hazard analysis, as part of Hazard Analysis and Critical Control Point (HACCP), to identify and to analyze microbiological and other hazards as-

Table 65–1 ICMSF Matrix of Stringency of Sampling Plan in Relation to Hazard and Conditions of Handling and Use*

	Conditions in Which the Food Is Expected To Be Handled and Consumed after Sampling		
Type of Hazard	*Reduced Concern*†	*No Change*††	*Increased Concern*§
No direct health hazard (eg, shelf life/spoilage)	Case 1 n = 5, c = 3	Case 2 n = 5, c = 2	Case 3 n = 5, c = 1
Health Hazard:			
Low indirect (indicator organisms) (eg, coliforms)	Case 4 n = 5, c = 3	Case 5 n = 5, c = 2	Case 6 n = 5, c = 1
Moderate direct (limited spread) (eg, *S. aureus, B. cereus)*	Case 7 n = 5, c = 2	Case 8 n = 5, c = 1	Case 9 n = 10, c = 1
Moderate direct (potentially extensive spread) (eg, *salmonella*, *shigella*)	Case 10 n = 5, c =0	Case 11 n = 10, c = 0	Case 12 n = 20, c = 0
Severe direct hazard (eg, *E. coli* 0157:H7; *S. typhi*)	Case 13 n = 15, c = 0	Case 14 n = 30, c = 0	Case 15 n = 60, c = 0

* Cases 1–9 are Three-Class Plans; cases 10–15 are Two-Class Plans; n = number of sample units tested; c = maximum allowable number of defective sample units (Two-Class Plans) or marginally acceptable sample units (Three-Class Plans). (For further information see Section 65.3.3.3).

† Reduced concern: eg, frozen raw crustacea cooked before consumption;†† No change: eg, chocolate;§ increased concern: eg, chill-stored, cooked meat products.

Source: Adapted with permission from ICMSF, *Microorganisms in Foods 2, Sampling for Microbiological Analysis: Principles and Specific Applications*, 2nd ed., © 1986, International Commission on Microbiological Specifications for Foods.

sociated with a particular food operation, and to design-in appropriate control measures (see Chapter 58).

A further need for formal MRA procedures arises from the recently implemented World Trade Organization Agreement on Sanitary and Phytosanitary Measures.[3] This requires a member government to base its laws for protecting human, animal, and plant health on an assessment of the risks involved and to be able to demonstrate that its national requirements are no more restrictive to trade than those required to protect the health needs of its subjects.

The Codex Alimentarius Commission's "Principles for the Establishment and Application of Microbiological Criteria for Foods" states that, "Microbiological criteria should be established according to these principles and be based on scientific analysis and advice, and where sufficient data are available, a risk analysis appropriate to the foodstuff and its use."[18,25] As Codex is recognized in the Sanitary or Phytosanitary (SPS) Agreement as the main source of international consensus on requirements for the production and distribution of safe foods, the principles for establishing microbiological criteria and any criteria derived from these would be expected to be accepted internationally. Additional information on the use of risk assessment as a foundation for establishing effective science-based food safety management systems is available.[14,37,38,53]

This chapter considers the developing links between microbiological risk assessment and microbiological criteria and reviews some of the options for objectively assessing microbiological risks and the use of microbiological criteria as one of the means of managing such risks.

65.2 RISK ASSESSMENT

65.2.1 Background

Risk assessment has been described as the process of organizing information concerning a particular risk in a systematic way so that decision makers can better understand those factors that contribute to the risk and any uncertainties that may affect the outcome of an assessment.[49]

The process of microbiological risk assessment has not progressed as far, or as fast, as other public health-oriented risk assessment procedures. The reasons why this process has proved more difficult than other such risk assessments include the inherent variability in virulence and growth characteristics of individual strains of specific microbial pathogens; their likely change in numbers in a food as it moves along the food supply chain; the often specific effects of foods and food components on virulence and survival of microorganisms through the intestinal tract;[4] and the idiosyncratic nature of the human response to infection.[1,9,23]

Microbiological risks associated with the consumption of food depend on the numbers, types, and virulence of organisms present in a food (or amounts of a microbially produced toxin or metabolite) at the time of consumption and the susceptibility of the person consuming the food to infection or intoxication by these. Thus any MRA must take account of microbiological knowledge concerning the likely occurrence and fate of microorganisms in a foodstuff, the possible influence of the food or the production process on the virulence, or survival of such organisms and/or their toxins, together with medical knowledge concerning the number of microorganisms or the amount of toxin estimated to cause illness, and the seriousness of such illness in terms of morbidity and mortality.[6]

The process of MRA generally involves four phases. The first is definition of the scope of the assessment (problem definition stage). The second involves collecting and organizing available relevant data and identifying important areas where data are absent (data collection and collation stage). The third involves analysis of the problem (problem analysis phase), a phase that will involve the use of some systematic process of risk assessment. In the fourth phase all the information is brought together to give an estimate of the probability of the occurrence of an adverse health effect (risk characterization phase).

Potential approaches to MRA include structured risk assessment techniques used in other disciplines. These include such techniques as Hazard and Operability Studies, Fault Tree Analysis, and Event Tree Analysis, these being used mainly for the evaluation of risks associated with chemical and physical hazards, including explosion risks and engineering failures associated with particular types of production plant.[1,40]

Further methodologies include Probabilistic Scenario Analysis[13] and the use of the paradigm used in formal and structured risk assessments of food ingredients, additives, and chemical contaminants based on a four-step procedure of Hazard Identification, Hazard Characterization, Exposure Assessment, and Risk Characterization.[15,23,63] The use of this latter procedure for MRA is being studied by a number of governmental bodies around the world (including the Codex Alimentarius Commission) with particular emphasis on attempting to use it to establish quantitative, or at least semiquantitative, risk assessment procedures.[24,32,63,71]

A drafting group of the Codex Committee on Food Hygiene met in 1996 and drafted a series of principles for applying MRA.[24] The following is based on the development of these principles by a working group of the European Union's Scientific Committee for Foods.[63]

- **Risk assessment must be soundly based on science**. Where scientific data are limited, otherwise incomplete, or conflicting, informed judgments should be made on the basis of the best available information.
- **Risk assessment should be separated functionally from risk management**. The latter is a separate process that is consequential on risk assessment and aimed at identifying options for action needed to manage an unacceptable risk and has a policy function.
- **A structured approach must be used when conducting a risk assessment of a microbiological hazard**. This structured approach should include Hazard Identification, Hazard Characterization, Exposure Assessment, and Risk Characterization; the order in which these steps are applied will depend on the assessment undertaken.
- **A risk assessment of a microbiological hazard must clearly state both the purpose of the assessment and the form of the risk estimate that will be the output**. The purpose may be, for example, to estimate the risk of a microbiological hazard in the total food supply or the microbiological hazards associated with a specific commodity. The output may be an estimate of the annual rate of illness in a population or the rate of illness per eating experience, etc.
- **The risk assessment must be transparent**. It must be fully documented, including any con-

straints that may have affected the quality of the risk estimate, and be freely available to independent assessors.

- **The risk estimate must contain a detailed description of uncertainty and where this arose during the Risk Assessment Process.**
- **The data used in a risk assessment must be of sufficient quality and precision that uncertainty in the risk estimate is minimized as far as possible.** Quantitative data should be used to the extent possible.
- **Where appropriate, a risk assessment of a microbiological hazard must consider the fate of the hazard(s) in food(s) and the disease process following infection.** It should consider the dynamics of microbial growth, survival, and death, and, where applicable, the dynamics of microbial toxin formation and destruction, together with distribution of the agent in the foodstuff(s) and interactions between the agent and the human body following consumption (including sequelae and the potential for secondary spread).
- **Risk estimates, where possible, must be reevaluated over time against human health data and when new data become available.**

A potential problem that has emerged during the initial attempts to develop a MRA process has been the confusion caused by the partial or total inclusion of the MRA procedure, as under development by Codex, into HACCP in an attempt to make the latter more quantitative.[13,55] While elements of HACCP include a risk estimate, it is important that the two processes are kept separate for the following reasons.

1. MRA, as defined by Codex, is a government-led procedure to be used by a regulatory authority to assess whether a particular microbiological hazard is an unacceptable health risk to a population, whereas HACCP is an industry-led procedure to be used by industry to identify and control the specific microbiological hazards associated with the production of a specific food or food ingredient using a specified procedure. These two separate processes are linked, as shown in Figure 65–1.
2. HACCP is based on a hazard analysis procedure leading to options for control based on hazard management (see Chapter 58), whereas risk assessment leads to the identification of control options based on risk management.[22]
3. The knowledge base and expertise needs of those involved in HACCP and MRA are quite different. Thus, factory personnel, microbiologists, and hygiene specialists would be involved in the development of a HACCP plan, whereas specialists with in-depth knowledge of a microbiological hazard, such as basic scientists, epidemiologists, and public health specialists would be involved principally in MRA. These latter specialists are unlikely to have the experience or knowledge to correctly identify the hazards and their control requirements in the many and varied food operations that exist throughout the food chain. Factory personnel, factory microbiologists, and hygiene specialists are unlikely to have the depth of knowledge concerning the biology of a pathogenic microorganism, and its role in foodborne disease, to properly estimate its risk as a contaminant in the human food chain.
4. To confuse the processes of risk assessment and HACCP would be prejudicial to their proper development, recognizing that they are distinct but interrelated procedures (Figure 65–1) for sound reasons (see Chapter 58).[66]
5. A HACCP team in a factory may use a published MRA as a source of information when conducting a hazard analysis of a food production operation. Those conducting an MRA, however, would not use the hazard analysis from HACCP plans as a resource material.

65.2.2 Definitions

Before further considering risk assessment procedures and their potential use in microbiological risk assessment, it is important to define some of the key words and phrases that are used because some of these differ from their common and expected usage.

When considering definitions, one is faced with a wide selection of possibilities, which is a reflection of the early state of development of this evolving branch of quantitative microbiology. One of the most recent attempts to establish an internationally accepted set of definitions was by a Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Consultation on the "Application of Risk Analysis to Food Standard Issues."[23] These definitions were later modified by the Codex Alimentarius Commission[21] and, although they are likely to be subject to further revision before becoming finally internationally accepted, they currently form a consensus of international opinion. They are used in the following

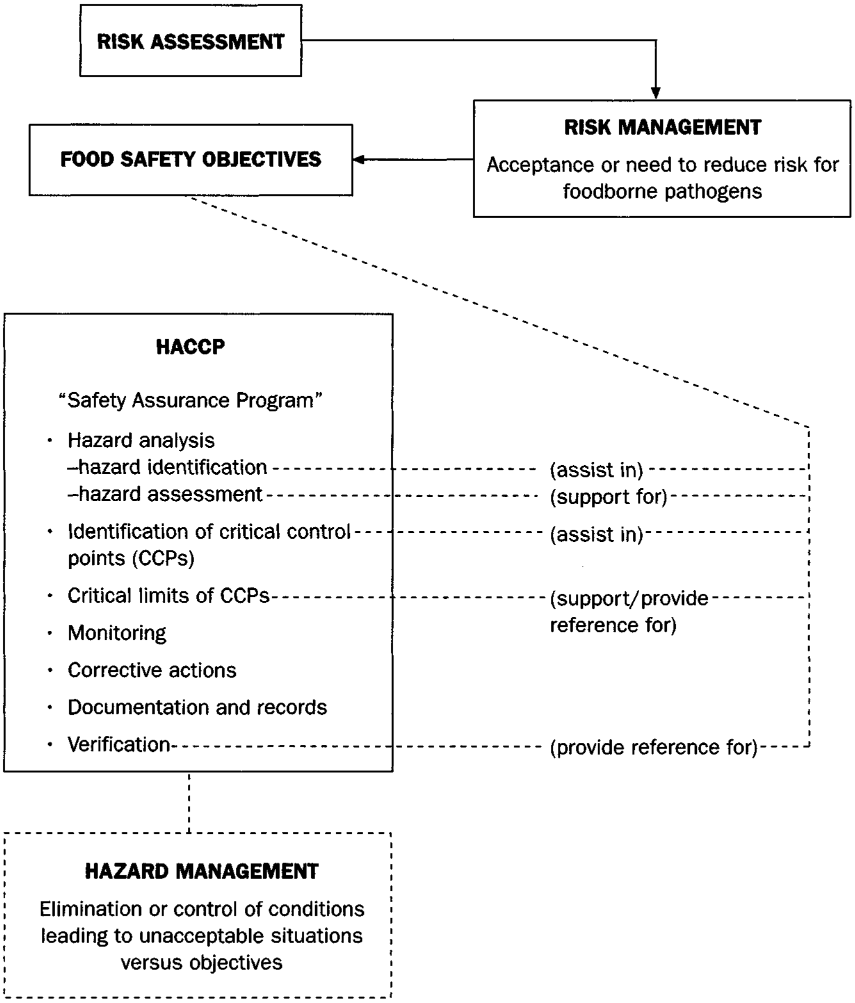
RISK ASSESSMENT
RISK MANAGEMENT
Acceptance or need to reduce risk for foodborne pathogens
FOOD SAFETY OBJECTIVES
HACCP
"Safety Assurance Program"
• Hazard analysis
–hazard identification
–hazard assessment
• Identification of critical control points (CCPs)
• Critical limits of CCPs
• Monitoring
• Corrective actions
• Documentation and records
• Verification
(assist in)
(support for)
(assist in)
(support/provide reference for)
(provide reference for)
HAZARD MANAGEMENT
Elimination or control of conditions leading to unacceptable situations versus objectives

Figure 65–1 Relationship of Risk Assessment and HACCP. Courtesy of J.L. Jouve.

paragraphs as the basis for discussion and are summarized in Exhibit 65–1.

Hazard. "A biological, chemical or physical agent in, or condition of, food with the **potential** to cause an adverse health effect."[21]

In most definitions, a microbiological hazard is defined simply as an inherent danger associated with a particular organism or toxin. In some sources, this simple definition is extended to include that to be a significant health concern, a certain number of organisms or an amount of a microbial toxin must be present. Thus the ICMSF[25] describes a microbiological hazard as **unacceptable** growth, survival, or contamination of a food with microorganisms and/or unacceptable production or persistence in foods of products of microbial metabolism such as toxins, enzymes, and biogenic amines. The International Life Sciences Institute Monograph on HACCP[39] combines the Codex definition with the ICMSF definition and states, "In the HACCP framework the term hazard refers to any agent in, or condition of, a food that is unacceptable because it has the potential to cause an adverse health effect." Here "condition" as in the Codex definition appears to refer to a condition arising as a result of contamination such as spoiled, adulterated, or unwholesome food, but this would seem to confuse the definition of a hazard. A recently completed document on HACCP by the US National Advisory Committee on Microbiological Criteria for Foods[51] defines a hazard as a "biological, chemical or physical agent that is reasonably likely to cause illness or injury in the absence of its control."

Exhibit 65–1 Definitions of Terms Applied to Risk Assessment and Hazard Analysis

Hazard: A biological, chemical, or physical agent in, or condition of, food with the potential to cause an adverse health effect.

Hazard analysis: The process of collecting and evaluating information on hazards, and conditions leading to their presence, to decide which are significant for food safety and therefore should be addressed in an HACCP plan.

Risk: A function of the probability of an adverse health effect and the severity of that effect consequential to a hazard(s) in food.

Risk assessment: A scientifically based process consisting of hazard identification, hazard characterization, exposure assessment, and risk characterization.

Hazard identification: The identification of biological, chemical, or physical agents capable of causing adverse health effects and that may be present in a particular food or group of foods.

Hazard characterization: The qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical, and physical agents that may be present in food. For chemical agents, a dose-response assessment should be performed. For biological or physical agents, a dose-response assessment should be performed if the data are obtainable.

Dose-response assessment: The determination of the relationship between the magnitude of exposure (dose) to a chemical, biological, or physical agent and the severity and/or frequency of associated adverse health effects (response).

Exposure assessment: The qualitative and/or quantitative evaluation of the likely intake of biological, chemical, or physical agents via foods as well as exposure from other sources if relevant.

Risk characterization: The qualitative and/or quantitative estimation, including attendant uncertainties, or probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization, and exposure assessment.

Risk management: The process of weighing policy alternatives in the light of the results of risk assessment and, if required, selecting and implementing appropriate control options, including regulatory measures.

Risk communication: The interactive exchange of information and opinions concerning risk among risk assessors, risk managers, consumers, and other interested parties.

Infection: Successful colonization of the host by a microorganism capable of causing damage to its host.

Source: Adapted with permission from FAO/WHO, *Codex Alimentarius Commission: Risk Assessment,* GL96/21 Gen. 1996, © 1996, Food and Agriculture Organization, and FAO/WHO, *Codex Alimentarius Volume 1B,* General Requirements (Food Hygiene) Supplement, © 1998, Food and Agriculture Organization.

Risk. "A function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard(s) in food."[21]

Severity is considered to be a component of risk by toxicologists and others;[49] however, most microbiologists also would consider severity as a component of a microbiological hazard. Thus, the presence of *C. botulinum* in an ambient-stable, low-acid canned food is a severe hazard but if the thermal process and other control measures are properly applied, the probability of this occurring is very, very low; therefore, the risk (ie, the likelihood) of botulism is extremely low. As a health concern must always take account of both seriousness and probability of occurrence, the overall conclusion as to whether or not there is a significant health concern does not change if severity is linked with risk rather than hazard. However, we believe that this is confusing and prefer to relate severity to hazard rather than to risk.

Risk Assessment. "A scientifically-based process consisting of the following steps: (1) Hazard Identification; (2) Hazard Characterization; (3) Exposure Assessment; (4) Risk Characterization."[21]

In outline it is a structured and formal procedure for identifying and characterizing a hazard and estimating (ie, quantifying) the effects of that hazard on a population.

> ***Hazard Identification.*** The identification of biological, chemical, and physical agents capable of causing adverse health affects and which may be present in a particular food or group of foods.

Hazard identification would include known hazards, such as those identified as a result of epidemiological studies of foodborne disease incidents, and potential hazards, such as those identified by research studies into the survival/growth characteristics of a pathogen in a foodstuff (see Section 65.2.3.1).

> ***Hazard Characterization.*** The qualitative and/or quantitative evaluation of the nature of the adverse effects associated with biological, chemical, and physical agents which may be present in food. For chemical agents, a dose-response assessment should be performed. For biological or physical agents, a dose-response assessment should be performed if the data are obtainable.

Hazard characterization provides an estimate of the severity and extent of illness likely to be caused by a particular number of microorganisms or an amount of a microbial toxin. The availability of soundly based dose response data is a particularly problematic part of hazard characterization because of the large number of factors that can affect this determination. Ideally, quantitative estimates are developed for the probability of an adverse health effect relative to the amount of agent consumed. This is discussed in detail in Section 65.2.3.2.

> ***Exposure Assessment.*** The qualitative and/or quantitative evaluation of the likely intake of biological, chemical, and physical agents via foods as well as exposures from other sources if relevant.

Obtaining meaningful microbiological data of numbers of a pathogen present in a food at the point of consumption is a difficult task because of the variable occurrence of pathogens in foods and the environment and large effect that processing, distribution, and preparation procedures for consumption can have on these numbers (see Section 65.2.3.3).

> ***Risk Characterization.*** The qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization, and exposure assessment.

This is the final step in the risk assessment procedure where the information obtained in the three previous steps is brought together into a statement of an estimate of microbiological risk(s) to a given population.

Risk Analysis. "A process consisting of three components: Risk Assessment, Risk Management and Risk Communication."[21,23]

It is important that the relationship between ***Risk Assessment***, ***Risk Management***, and ***Risk Communication*** is understood. ***Risk Assessment*** is the four-step procedure described above and results in a risk estimate. ***Risk Management*** is the separate process of weighing policy alternatives in light of the results of risk assessment and selecting and recommending control options if the risk needs to be controlled. It consists of the processes of Risk Evaluation, Risk Management Option Assessment, Implementation of Management Decisions, and Monitoring and Review.[22] ***Risk Communication*** is the exchange of information and opinion interactively among risk assessors, risk managers, and other interested parties. These two latter activities are outside the scope of this chapter, but the use of a microbiological criterion, which will be considered later, is an example of the application of a Risk Management option. Also, any regulation or guideline that incorporates such a microbiological criterion would be a form of risk communication. The interrelationship between Risk Assessment, Risk Management, and Risk Communication is shown in Figure 65–2.

Hazard Analysis. "The process of collecting and evaluating information on hazards, and conditions leading to their presence, to decide which are significant for food safety and, therefore, should be addressed in the HACCP plan."[25]

It is particularly important to recognize the specific use of the words hazard, risk, risk assessment, risk analysis, and hazard analysis in the following sections on microbiological risk assessment and microbiological criteria.

65.2.3 Status and Application

The following section reviews the status of the basic knowledge needed for a microbiological risk assessment and examples of its application. It will consider this knowledge base against the needs of the four-step risk assessment process described above and it will attempt to identify what new information will be needed to make this type of risk assessment possible.

65.2.3.1 Hazard Identification

Knowledge of microbiological hazards is derived mainly from accounts of epidemiological investiga-

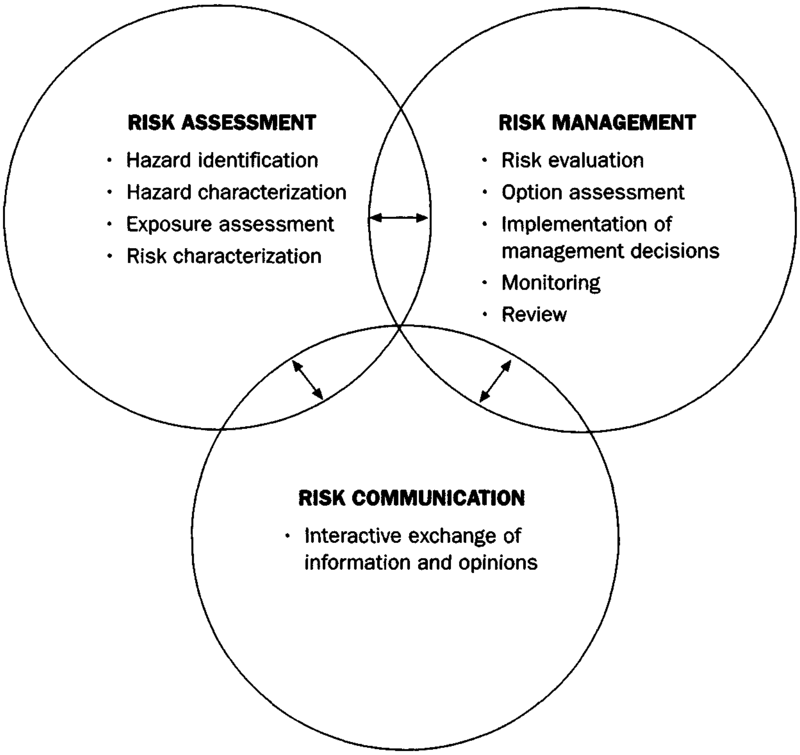

Figure 65–2 Risk Analysis Framework. *Source:* Adapted with permission from SCF, *Principles for the Development of Risk Assessment of Microbiological Hazards for the Development of Hygiene Risks for Food Stuffs as Covered by the Hygiene of Foodstuffs Directive 93/43/EEC.* Scientific Committee for Foods (SCF) DG 24, © 1997, European Commission, Brussels.

tions and records of incidents of foodborne illness. Additional information may be derived from clinical studies. The uses and means of obtaining epidemiological and clinical information are described in Chapter 37, together with the limitations of such information. Briefly, our knowledge of the extent and many of the causes of foodborne illness is very incomplete as there is gross underreporting of such illness in all countries. Even when there is a report of an illness with symptoms that suggest that it may be foodborne, the cause of the illness is often unknown and the food involved seldom identified.[7,8,59,69,78]

The reasons for underreporting, in addition to the often lack of an effective reporting scheme, include failure of an ill person to seek medical help, failure of the clinician to report a case of suspected foodborne illness, absence of suitable samples for analysis, and the cost and difficulties involved in investigating an incident. Cost is particularly a constraint when an incident may involve only one or two cases of a distressing, but not life-threatening, illness. Thus, only incidents involving multiple cases (ie, outbreaks) are investigated in sufficient depth to provide data that can be used for hazard identification. Such data should include causative agent (amount or number involved); source of agent and circumstances that lead to it causing illness; type(s) of foods involved; extent of illness and persons involved (age, health status); and type of illness caused including time from consumption, symptoms, severity, and duration.

Additional factors that may influence the identification of a new etiological agent of foodborne illness include the availability of detection methods and the effect of incubation time. In the former, a number of years usually will elapse between identification of a new agent and the development of reliable and sensitive methods of detection. In the latter, acute illness with a short incubation time among a group of people eating a common meal would be more likely to be recognized as an outbreak than if the symptoms required days for onset and the participants in the meal are widely dispersed. Microorganisms causing outbreaks always will be more recognized as a health concern than those only causing sporadic cases. This can be illustrated by the fact that listeriosis only finally was recognized as a foodborne disease when an outbreak occurred in Canada in 1981, despite the fact that sporadic cases had been reported a long time before this. Campylobacter enteritis, which is now recognized to be one of the most common causative agents of foodborne illness, similarly was not identified for many years because cases are almost entirely sporadic and detection methods were not available or were not in use.

The continued generation of sound epidemiological and microbiological data is essential if we are to be able to effectively identify new microbiological hazards. The erosion in many countries of the expertise and facilities to carry out epidemiological investigations, mainly as a result of loss of funding, and reduced ability to perform the underpinning microbiology research, needs to reassessed in the light of the clear need for this basic information for MRA. As part of this ongoing activity, there is a need for epidemiological surveillance of illness within populations to identify potential new problems and to better quantify the extent of existing ones; sentinel studies are particularly useful in this regard (see Chapter 37). Also, targeted surveillance of foods for specific microorganisms needs to be an ongoing activity recognizing that new foods, new food sources, and new processing and packaging methods will continue to be developed to meet the changing tastes and expectations of consumers throughout the world—activities that may give rise to new microbiological hazards.

Further, all existing knowledge on microbiological hazards needs to be incorporated into readily accessible databases that can be updated as new information becomes available; the Internet is proving to be a useful vehicle for the rapid dissemination of such information. Even in Western countries, much potentially useful epidemiological and surveillance data are not currently accessible to those concerned with the use of such information for hazard identification.[1] The WHO (European region) coordinates and disseminates information on the types of microbial agents reported to cause foodborne illness in many European countries.[78]

Knowledge of factors affecting the growth, survival, and death of established and emerging food pathogens in foodstuffs, and basic studies of their genetics, physiology, and biochemistry are essential for hazard identification and characterization. Considerable progress has been made in recent years in the development of more accurate mathematical models predicting the growth, survival, and death of microorganisms in foods and developing means of making this information widely available in the form of portable computer databases (see Chapter 16). Further development of electronic databases, combining these models with other information on the biology and disease characteristics of these organisms into expert systems, will considerably enhance the usability of these data for nonexpert hazard identification, a particular need for use in the hazard analysis stage of HACCP.

Summary. There is a need to improve the collection and dissemination of information on the microbiological agents involved in foodborne illness, the foods involved, and the circumstances that led to illness. Such epidemiological information should be collected by health authorities throughout the world and made available to national and international bodies concerned with risk assessment.

65.2.3.2 Hazard Characterization

The goal of hazard characterization is to provide a quantitative, or at least a semiquantitative, estimation of the severity and extent of illness caused by the presence of a particular number of microorganisms or an amount of a microbial toxin in a foodstuff at the point of consumption. Thus, when considering hazard characterization, there is a need to consider the availability of dose response data (ie, the probability of illness and the degree of illness caused by a particular concentration of a microorganism or toxin). Specifically, the risk of infection has been defined as the mathematical probability of infection from a given dose of exposure.[61]

There are a number of ways in which knowledge can be obtained of the infective dose. The classic way, and considered by many to be the best, is the use of controlled challenge studies in which a statistically significant group of human volunteers is fed different concentrations of microorganisms and the clinical symptoms observed. This method has been used to generate dose-response data for a range of microorganisms.[68] These have been used in "model" risk assessments mainly by those concerned with the control of microbial diseases that are spread by water or are associated with the consumption of aquatic animals such as mollusks.[30,31,46,58,60,61,62]

Many of the published dose response data have been based on feeding healthy adult, male volunteers (mainly prisoners or students).[68] However, data obtained from incidents of foodborne illness indicate that many of these data give low estimates of the probability of illness from particular concentrations of pathogenic microorganisms. For example, results of feeding trials using salmonellas suggest that a minimum dose of 10^5 is necessary to cause illness, whereas there is much evidence that under some circumstances widespread illness can result from the consumption of as few as 10 cells (see Chapter 45). Some of the reasons for such differences, in addition to age and health status, are listed in Table 65–2 and include microbiological factors such as variability in the virulence of strains within a particular microbial species, the influence of a particular food type, or other microorganisms on survival through the intestinal tract or on the production of virulence factors. Important factors affecting the susceptibility of the host include health, nutritional and immunological status of the individual, status of digestive tract at time of consumption of a contaminated food, and the possession of certain human antigenic determinants in the body fluids.

A further method of obtaining dose response data is to collect information on the number of a microorganism/amount of toxin present in a food implicated in a foodborne illness incident, and to relate such information to the percentage of persons with symptoms and the seriousness of the symptoms observed. The information generated by this means is likely to be more realistic than that generated by volunteer studies but most of the same constraints apply, as do some additional ones—for instance, the poor accuracy and reliability of the microbiological data obtained from the analysis of a food, sometimes days or even weeks after the food caused illness, recognizing that the food may have been stored under conditions allowing microbial numbers or an amount of a microbial toxin to change. Further, while such data may give some information on the minimum infective dose (MID) in a particular food, it is unlikely to provide dose-response data or data relating infectivity or severity of illness to dose. Data relating dose to severity may be obtained by statistically analyzing the results of a number of separate incidents involving the same organism. Thus Glynn and Bradley[29] analyzed epidemiological data from 68 outbreaks of typhoid and 49 outbreaks of salmonellosis. Based on reported severity (ie, number of patients hospitalized compared with total number affected), they concluded that there was a dose-response relationship for severity of disease caused by nontyphoid-causing salmonellas but not by *Salmonella typhi*; a possible reason for not demonstrating a relationship for *S. typhi* is the severity of illness caused by low doses of this organism.

Through the methods outlined above (ie, feeding trials and retrospective analysis of outbreak data), it is possible to develop probability models to estimate the risk of infection resulting from exposure to different concentrations of microorganisms. The development of such models for foodborne pathogens is still a relatively new development, although the beta-distributed infectivity probability model or simply the Beta-Poisson model, which is the basis of most such models, was described in the early 1980s by Haas.[30,31]

The Beta-Poisson model is described by the equation $P_i = 1-(1+(N/b))^{-a}$; where P_i is the probability that an individual exposed to an average dose of N organisms will become infected, and a and b are parameters that define the dose response curve.[30,31,61] This model was found to most closely fit dose-response data from human feeding trials.[31] Two other models, a log normal and an exponential model, examined by Haas did not so closely fit the feeding trial data. The Beta-Poisson model assumes that a single bacterium or virus particle is capable of initiating infection, that there is a Poisson distribution of cells or particles in replicate doses, and that there is a distribution of probabilities that an agent, once ingested, will survive and initiate infection, a process that is mathematically described by the beta distribution.

The use of the beta-distribution infectivity model to generate data on the probability of infection resulting from exposure to different concentrations of microorganisms has been criticized because it makes several assumptions that are not likely to be correct. The first is that it assumes a random (Poisson) distribution of microorganisms in a water source or foodstuff (a possibility for the former but highly unlikely for the latter); the second is that susceptibility to illness is normally distributed in a population, which is highly unlikely for the reasons listed in Table 65–2. Despite these limitations, some interesting probability estimates of illness have been obtained.[30,61,68] An example of one of these estimates is shown in Table 65–3.

Table 65–3 illustrates the effects of dose (quantity of infectious agent) and the relative virulence of two pathogens (rotavirus and Hepatitis A virus [HAV]) on the probability of infection, illness, and death among a population consuming shellfish.[61] To arrive at their estimate, the following was assumed:

- the Poisson distribution of virus particles in the shellfish
- the shellfish was consumed raw and 60g (six small shellfish) represented a single serving
- the enterovirus levels measured in various surveys of shellfish could be averaged to represent

Table 65–2 Factors Increasing the Risk of Foodborne Infection or the Severity of Illness

Factors	*Reasons*
Microbial factors	
Type and strain of pathogen ingested	Some pathogens and strains more virulent than others
Quantity of pathogen ingested	Higher numbers ingested may increase severity of illness and/or shorten onset time
Host factors	
Age less than 5 years	Lack of developed immune systems, smaller infective dose-by-weight required
Age greater than 50 or 60 years (depending on pathogen)	Immune systems failing, weakened by chronic ailments, occurring as early as 50 to 60 years of age
Pregnancy	Altered immunity during pregnancy
Hospitalized people	Immune systems weakened by other diseases or injuries or at risk of exposure to antibiotic-resistant strains
Concomitant infections	Overloaded or damaged immune systems
Consumption of antibiotics	Alteration of normal intestinal microflora
Excessive iron in blood	Iron in blood serving as nutrient for certain organisms
Reduced liver/kidney function (alcoholism)	Reduced digestion capabilities, altered blood-iron concentrations
Possession of certain human antigenic determinants duplicated or easily mimicked by microorganisms	Predisposition to chronic illnesses (sequelae)
Surgical removal of portions of stomach or intestines	Reduction in normal defensive systems against infection
Immunocompromised individuals including those on chemotherapy or radiation therapy; recipients of organ transplants taking immunocompromising drugs; persons with leukemia, AIDS, or other illnesses	Immune system inadequate to prevent infection
Stress	Body metabolism changes allowing easier establishment of pathogens, or lower dose of toxin required for illness
Poor hygiene	Increased likelihood of ingestion of pathogens
Diet related factors	
Nutritional deficiencies either through poor absorption of food (mostly ill or elderly persons) or unavailability of adequate food supply (starving people)	Inadequate strength to build up resistance and/or consumption of poor-quality food ingredients, which may contain pathogens
Consumption of antacids	Increased pH of stomach
Consumption of large volume of liquids, including water	Dilution of acids in the stomach and rapid transit through the stomach
Ingestion of fatty foods (such as chocolate, or cheese) containing pathogens	Protection of pathogens by the fat against stomach acids
Other factors	
Geographic location	Likelihood of exposure to endemic virulent strains, limited food and water supply, varied distribution of organisms in water and soil

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an average exposure for a variety of enteric viruses

- the echovirus and rotavirus dose-response models represented moderately and highly infectious viruses, respectively; a previous model of echovirus-12 was used to develop the estimate for risk of infection by HAV.

The data indicated that as the level of either virus increased in the 60g serving of shellfish the risk of infection, illness, and death increased. The risk of infection was higher for rotavirus, but the risk of death is slightly higher for HAV. However, these data do not bring out other factors that are important in the pattern of illness shown by these two viruses. For

Table 65–3 Risks of Infection, Morbidity, and Mortality for Consumption of Viral-Contaminated Raw Shellfish for a Single Exposure[*]

	Risk of Infection		*Risk of Illness*		*Risk of Death*	
Viral Levels 60 g^{-1}[†]	*RV*[‡]	*HAV*[§]	*RV*	*HAV*	*RV*	*HAV*
000.1	0.054	0.0002	0.032	0.00015	0.000003	0.0000009
1.0	0.27	0.002	0.16	0.0015	0.000016	0.000009
10.0	0.57	0.02	0.34	0.015	0.000034	0.00009
100.0	0.76	0.15	0.46	0.11	0.000046	0.00066

[*] The risk is the fraction of the population who are likely to experience the consequences (infection, illness, or death).

[†] Single serving of six shellfish.

[‡] RV = rotavirus.

[§] This assumes that the infectivity of Hepatitis A virus (HAV) is equal to the Echovirus-12 model, which is more moderate than the rotavirus. The mortality for HAV, however, is much more significant than for rotavirus.

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example, the risk of illness from HAV is lower among children than among adults, whereas the risk of illness from rotavirus is higher among children.[20,28] The reason for this is host resistance (eg, immune status) and is an example of a model not containing complete information on factors affecting infectivity (Table 65–2) that may lead to misleading conclusions. It is important that such modeling of dose and risk is further developed to take account of these additional factors as this information is essential in the assessment of microbial risk.

The final type of information to consider is the use of expert judgment to obtain an estimate of the likelihood and extent of illness when exposed to different concentrations of microorganisms. The publication of part of a large US Food and Drug Administration study[47] is illustrative of this approach. In this study, a group of experts on foodborne pathogens was asked to consider the likely outcome, in terms of absence of illness, or where illness occurs the severity of illness, when a population is exposed to different concentrations of foodborne pathogens. The data obtained were subjected to computer analysis to estimate variances in responses obtained and to quantify the uncertainty associated with each probability judgment. This technique has some possibilities, but the results obtained demonstrated the considerable divergence of opinion among experts, even when they are given the same information and asked to use it as the basis of their estimates. It was concluded that this technique is not a substitute for actual data on dose-response relationships.

The use of animals for hazard assessment has not been discussed because the consensus view among experts on microbial disease assessment is that extrapolation of animal dose-response data to humans is not useful because of the highly variable response of animal models, the exception being primates. Animal models are, however, useful for studying disease mechanisms.

The use of the concept of a MID for a microorganism as an expression of the lowest number of microorganisms able to cause infection, and hence potential to cause disease, is currently regarded to be of limited use for hazard characterization of a pathogenic microorganism.[1] First, in practice, it is impossible to measure, and second, it seems reasonable to assume that each cell of a pathogenic microorganism has the potential to initiate infection and to cause disease in an individual; the greater the virulence of a microorganism, the greater this likelihood. Whether or not infection and subsequent disease occur depends on the number of individuals who are exposed to the organism (ie, the greater the number, the higher the probability that an organism will be ingested by a sensitive individual and cause infection). In risk assessment, it is the risk of infection in a given population or subpopulation that is of main concern, ***not*** the risk of infection of an individual.

Summary. There are a number of practical means of collecting data suitable for the characterization of a microbiological hazard but all have limitations. There is a need to develop better data and dose-response models that can take account of the many factors affecting infectivity and the development of foodborne illness.

65.2.3.3 Exposure Assessment

The aim of exposure assessment is to evaluate the degree of human exposure to a microbial pathogen or toxin that is likely to occur as a result of food consumption. Thus it concerns obtaining knowledge of the numbers and types of foodborne pathogens and microbial toxins that may be present in food(s) at the point of consumption. It must consider changes in numbers of pathogens present in raw materials and/or products or introduced as a result of contamination during processing, storage, or preparation for consumption. With regard to toxins, consideration must be given to the conditions necessary for toxin production pre- and postharvesting and during any manufacturing or distribution procedure. Conditions leading to destruction of toxins during processing or preparation of a food for consumption must also be considered.

Data on the occurrence of foodborne illness-causing microorganisms in raw and processed foodstuffs have been collected and analyzed by many bodies. What they broadly indicate is that virtually any animal, plant, or environment-occurring pathogen may be found on raw agricultural products with variable and largely unpredictable frequency and amount. This will depend on where the product is grown, how well it is protected from contamination during growing and harvesting, and how well it is handled during subsequent handling prior to consumption. With most processed foods, much less variation will be found, provided they are produced under conditions where Good Manufacturing Practices (GMP) and HACCP are properly applied and storage conditions are well controlled. Data on the occurrence of microorganisms on foods after manufacture can be obtained by the surveillance of foods in the marketplace. However, in order that useful results are obtained from a surveillance program, it is important that statistically based sampling plans are used that take account of the likely frequency of occurrence of the target pathogen(s) in the foods under surveillance, and that the foods sampled are representative of those to which a population would usually be exposed; for further information on the choice of sampling plans for the surveillance of foods for microbiological contaminants, see Chapter 60.

Computer-based mathematical models that predict microbial growth and death in foods when subjected to changing conditions of temperature, for example, are potentially useful for studying the patterns of growth and survival of pathogenic microorganisms in foods throughout the food chain and therefore may provide predictions of numbers of microorganisms in a food at the point of consumption (see Chapter 16).[10,48,50,57,76] However, information on numbers of microorganisms in raw materials, introduced during processing, together with conditions such as temperatures likely to be encountered by a food during distribution, or retailing, will not be generally available and will severely limit the use of such models in exposure assessments. Also, many of the conditions affecting the growth and survival of microorganisms in foodstuffs have yet to modeled. Thus, any estimates of exposure made using such models would need to be checked against actual data. Predictive models are much more useful in HACCP studies than exposure assessments.

Other practical problems limiting the accuracy of exposure assessments include:

- failure to detect microorganisms damaged in some way so that they are not detectable using standard microbiological techniques;
- general heterogeneity in the distribution of microorganisms in foodstuffs making their accurate estimation difficult;[42]
- many potential sources of microbial contamination of foods as they proceed along the food chain, making estimates of likely numbers in foods at point of consumption even more inaccurate;
- changing population demographics and consumption patterns of foods; and
- introduction of new foods, new preservation systems, and new cooking procedures (such as microwave cooking) that may change the degree of exposure of the food consumer to particular pathogens (eg, psychrotrophic pathogens such as listeria, yersinia, and nonproteolytic *C. botulinum* in extended shelf-life, chill-stored foods).

In the absence of sound data on exposure of a foodborne pathogen to a population, a regulatory authority making a risk assessment will wish to err on the side of caution. Thus, when in the early 1980s *Listeria monocytogenes* caused several large outbreaks of listeriosis, several regulatory agencies introduced regulations requiring the absence of the organism from ready-to-eat foods. However, when it was subsequently found that *L. monocytogenes* was a common contaminate of food, a number of these authorities[26,67] revised their risk estimates, accepting that for most foods intended for general consumption, total absence was not necessary to protect the health of the consumer.

Summary. Exposure assessment of foodborne pathogens and toxins is difficult, and largely uncertain, because of the numerous opportunities throughout the food chain for microbiological contamination to occur; unprocessed foods are particularly subject to contamination by a wide range of pathogenic microorganisms. The heterogeneous distribution of pathogenic microorganisms and their toxins in most foodstuffs makes the accurate estimate of amounts difficult, and their changing concentrations with time make estimates of exposure at the point of consumption even more difficult.

65.2.3.4 Risk Characterization

Risk characterization is the final step of the risk assessment process, where all the information collected and collated in the three previous steps is assessed. Its aim is to give an estimate of the probability of occurrence of illness (including a description of any statistical and biological uncertainties) within a given population, taking into account the severity of illness likely to be caused, the number of organisms or the amount of toxin needed to cause illness (hazard characterization), and an assessment of the likelihood of exposure of members of the population (exposure assessment).

By way of example of applying risk characterization and the potential use of the four-step process of risk assessment to assess microbial risks, we will consider three examples of microbiological risk assessment.

The first is one of a series of examples presented by Todd and Harwig[71] of model risk assessments of foods distributed in Canada. To identify the microbiological hazards associated with meat and poultry products (hazard identification), they reviewed the available epidemiological information (number of cases, severity of illness, sections of population particularly at risk) on illnesses arising from the consumption of these foods and selected those organisms they considered to be most significant in terms of human morbidity and mortality. These were salmonellas, campylobacters, verocytotoxigenic *Escherichia coli*, *L. monocytogenes*, and *Toxoplasma gondii*.

For the second step of the assessment process (hazard characterization), they selected salmonella and toxoplasma to serve as examples. For salmonellas, they collected information on contamination rates on different meat products in Canada, including information on the percentage of carcasses and cuts contaminated, and sources and routes of contamination. They established an average contamination rate for poultry of 75% and for other meats of 5%. Contamination levels on raw meats were assumed to be low (eg, for poultry a level of 1 to 30 cells cm^{-2} was used based on data established by other workers). They did not estimate reductions achievable by cooking or the potential for recontamination, but recognized that these were additional factors to be considered in a risk assessment.

They estimated that 90% of the population of Canada (ie, 27 million) consumed chicken and other meat products. Thus, they calculated that the population exposed to salmonella from poultry is $75\% \times 90\% \times 27 = 18.2$ million and for raw meats $5\% \times 90\% \times 27 = 1.2$ million. This is an oversimplification of exposure as it does not take into account frequency of consumption of the different meats, or amounts consumed by different population groups or how the products might be prepared for consumption.

Although Todd and Harwig did not recognize a risk characterization stage in their analysis, they did attempt a risk estimate for each of the organisms they considered. Thus, based on other data, they stated that about half of the estimated 627,200 cases of salmonellosis in Canada each year arise from the consumption of meat, with about 36% of cases from poultry and 12% from other meats; this gave an estimated number of poultry- and meat-derived cases to be 227,000 and 76,000, respectively. From the information on the population exposed to salmonellas, they calculated the ratio of ill to at-risk cases for poultry meat as 1 in 80 (227,000/18.2 million) and for other meats as 1 in 16 (76,000/1.2 million). This led to the interesting conclusion, based on their assumptions and estimates, that although red meats are 15 times less likely to be contaminated with salmonella than poultry meat, they are 5 times more likely to cause salmonellosis. One possible explanation for this could the tendency to undercook red meats. If so, a risk management strategy derived from such a study might be to encourage more thorough cooking of these products.

A second example of the application of the risk assessment procedure is an estimate of the risk of viral infection from consuming raw shellfish harvested from approved waters in the United States.[61] As discussed previously (Section 65.2.3.3), probability estimate models for echovirus-12 and rotavirus were used to assess the risk of an individual becoming ill after consuming a single serving of shellfish. The estimated risk of illness was 1 in 100 if echovirus-12 (a moderately infectious virus) was present; this increased to 5 in 10 if a highly infectious virus, such as rotavirus, was present at a concentration of 100 virus particles/serving.

In a third example of risk assessment, the public health significance of cracked eggs was assessed.[70] Based on epidemiological information and literature searches, it was concluded that *Salmonella* is the only pathogen that has been conclusively linked with illness from cracked eggs. Of the eggs graded in Canada, about 2.5% were in the category that includes cracked eggs. It was estimated that there are approximately 143 million cracked eggs produced in Canada each year, and an estimated 10,500 cases of salmonellosis resulted from their consumption. Cracked eggs were estimated to be between 3 and 93 times more likely to cause salmonellosis than uncracked eggs. On average, the risk of illness from consuming contents of cracked eggs was estimated to be 1 in 3,800; however, some sectors of the population would be at a higher risk. A detailed risk assessment of *S. enteritidis* in shell eggs and egg products is contained in a recent US Department of Agriculture (USDA) report.[5]

A number of risk assessments have been described recently using computer simulation models to predict the effect of processing and storage regimes on microbial growth and survival in specific foods.[10,48–50,57,76,77] These models take information on the concentration and distribution of a particular microorganism in, for instance, a raw material and based on knowledge of the effect of processing and storage conditions are able to predict likely changes in numbers from a production stage to consumption and to predict the number of organisms that could be consumed.

The most recent of these risk assessments[10,57,77] interface such predictions with infective-dose models and are thus able to predict the chance of infection from consumption of a portion of food contaminated with a particular concentration of a microorganism. However, as noted previously, the amount of specific data required to use such models is very high. This includes, for instance, knowledge of the range and distribution of the target microorganism in a material to be processed and the kinetics of any destruction process or condition permitting growth to occur during or after a production process.[10,50] Thus their use appears limited mainly to specific food operations. Here, they are able to provide quantification to the hazard analysis process of HACCP as well as being useful to explore the impact of different process and product formulation options on the microbial risks of manufactured foods.

Summary. Microbiological risk assessment is a fast-developing field of quantitative microbiology. Estimates of risk are likely to be inaccurate for the reasons explained; however, the four-step risk assessment procedure described can be used to semiquantify, rank, and compare microbiological risks.

65.3 MICROBIOLOGICAL AND RELATED CRITERIA

65.3.1 Background

Several different types of criteria are used by food microbiologists to manage and control microbiological risks. The first and most commonly used are ***Microbiological Criteria***, which are used as the basis for deciding whether the concentrations of specific and general types of microorganisms and toxins measured in a foodstuff are acceptable or not. Such criteria may be classified into microbiological standards, specifications, and guidelines to reflect their different and varied use by industry and the regulatory authorities as risk management tools. They are applied usually to ingredients and finished products and less commonly to foods while in the process of manufacture.

The second group of criteria are ***Performance*** and ***Process Criteria***. These are applied during the production of a food with the aim of building into the manufacturing process effective measures to control the risks of identified microbiological hazards to acceptable (safe) levels.[37] A performance criterion has been described as the outcome of one or more control measures at a step (eg, operation, processing step, or stage in the food chain) or a combination of steps intended to ensure the safety of a food. For example, a 12D kill of *Clostridium botulinum* spores in a sterilized canned food, or defined composition and/or storage conditions (eg, formulation, time and temperature of storage, type of packing) of a perishable food to prevent a 1 log increase in growth of a pathogen. Process criteria are the control parameters (ie, the specific temperature, time, pH, a_w, etc.) that are applied at a particular step or a combination of steps to meet the performance criterion. For instance 2.5 minutes at 121° C to achieve a 12D kill of *C. botulinum*, a pH of 4.6 or less in a canned food to prevent the growth of *C. botulinum* in an acidified

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canned food. Process criteria may be used by food producers as control measures (Critical Limits) at specific critical control points (CCPs) and thus may be widely applied in HACCP plans.

Both performance and process criteria may also be specified by regulatory agencies as mandatory or advisory control measures. For example, many regulations specify a heat treatment for the pasteurization of milk at 71.7° C for 15 seconds. There is growing interest among regulators in some countries to establish further process and performance criteria based on risk analysis and to require industry to design and manage their processes to meet these through the application of GMP and HACCP. Examples include performance criteria for juice products and meat and poultry products.[27,75]

The following are examples of the application of microbiological, performance, and process criteria; they are intended to demonstrate the relationships between these types of criteria as control measures. The first is a series of options for the control of *C. botulinum* in preprepared chill-stored perishable foods recommended in a report by the UK Advisory Committee on the Microbiological Safety of Food.[2] These options were:

1. A heat treatment of 90° C for 10 minutes or equivalent lethality
2. A pH of 5 or less throughout the food and throughout all components of complex foods
3. An a_w of 0.97 or less throughout the food and throughout all components of complex foods
4. A combination of heat and preservative factors that can be shown consistently to prevent growth and toxin production by *C. botulinum.*

Option 1 is a process criterion directed toward killing vegetative cells and spores of psychrotrophic strains of *C. botulinum* that may be present in the raw materials used in the production of the foodstuff. Options 2 and 3 are further process criteria intended to prevent the growth of vegetative cells of this organism and hence prevent toxin production. Option 4 could involve thermal destruction/thermal injury of spores and inhibitory factors to prevent the outgrowth of surviving *C. botulinum* spores.

Underlying each process criterion option is an unstated performance criterion. Thus the performance criterion for option one could be stated as a 6D reduction of spores of psychrotrophic strains of *C. botulinum* as this is the intended result of a heat treatment of 10 minutes at 90° C. For options 2 and 3, the performance criterion could be stated as less than 1 log increase of *C. botulinum* within the use-by date when stored at the recommended storage temperature. Thus performance criteria do not describe how to control risk, but rather specify the end result that must be obtained. Process criteria specify the control parameters that can be applied to achieve the performance criteria.

The second example concerns the risk of *E. coli* O157:H7 and similar foodborne pathogens in fermented sausages. In December 1994, an outbreak of foodborne illness caused by *E. coli* O157:H7 occurred on the West coast of the United States (US) that was attributed to a fermented sausage product. In response, the USDA proposed a requirement that all manufacturers must use processes that will control the risk of illness from *E. coli* O157:H7. In this case, the agency proposed a performance criterion (ie, 5D kill of *E. coli* O157:H7) and left it to industry to decide how to satisfy the criterion and still produce products of acceptable quality. The agency's proposal of a 5D kill was based on very limited evidence suggesting that up to 1,000 g^{-1} of *E. coli* O157:H7 could occur in the raw meat used for processing. On the basis of industry-sponsored research, five options were developed and accepted by the agency.[54]

1. Apply an existing, approved heat treatment specified in USDA regulation 9CFR 318.17 (ie, heating to an internal temperature of 62.8° C or to a lower temperature for such time necessary to obtain an equivalent level of safety).
2. Apply a process that is validated by research to cause a 5D kill of *E. coli* O157:H7 before the product is released for distribution.
3. Combine raw material testing with a process that is validated by research to cause a 2D kill of *E. coli* O157:H7 before the product is released for shipment. The sampling procedure must ensure the level of *E. coli* O157:H7 in the raw sausage blend does not exceed 1 g^{-1}. One such sampling plan could consist of analyzing 15 sample units (25g each) collected at the same time the sausage blend is stuffed into the casings.
4. Apply a hold and test program for the finished product before distribution. Products intended to be heated before serving (eg, pepperoni for pizza) would be sampled at a rate of 15 sample units per lot. Products normally consumed without heating before serving (eg, salami) would be sampled at a rate of 30 samples per lot. The analytical unit for each sample unit tested would consist of 25g.
5. To allow for new technology or ideas, this option permits the use of alternate processes that

ensure the level of *E. coli* O157:H7 will not exceed 1 100 g^{-1}.

All five options are intended to yield no greater than 1 *E. coli* O157:H7 $100g^{-1}$ when the products are released for distribution. This was considered by the USDA at the time to be an acceptable level of risk for this class of product. The five options include process criteria, performance criteria, and microbiological criteria. Options 1 and 2 assume an initial level of 1,000 *E. coli* O157:H7 g^{-1} in the raw sausage blend. The process criterion for option 1 (heating to an internal temperature of 62.8° C) is derived from an existing regulation for roast beef and is based on research data demonstrating that this will result in a 5D kill of *E. coli* O157:H7 in beef; processors choosing this option would not take advantage of the faster rate of kill that would occur with the reduced pH of a fermented product.

The performance criterion in option 2 specifies a 5D kill of *E. coli* O157:H7. To satisfy this option, the processing plant must have on file, and available for review, research data that validate that the process being used will achieve the 5D kill; the research protocol for validating the process must be approved by the USDA.

Option 3 makes use of both a performance and microbiological criterion. Thus it involves a performance criterion of a 2D kill, in combination with microbiological testing to verify that the level of *E. coli* O157:H7, in each production lot, does not exceed 1 g^{-1} in the raw sausage blend; collectively, the net result is equivalent to the 5D kill of option 2, which assumes an initial level of 1,000 g^{-1} in the raw sausage blend.

Option 4 establishes microbiological criteria for the product and assumes no prior knowledge of the level of *E. coli* O157:H7 in the raw sausage blend or the lethality of the process. Reliance is placed solely on the use of a sampling plan that may detect *E. coli* O157:H7 if it is present in the finished product.

It is an historical fact that the major advances in public health have been made by applying interventions, such as the use of milk pasteurization or water chlorination to control specific microbiological hazards, ie, application of performance and process criterion. We are not aware where significant foodborne hazards to health have been reduced through the application of a microbiological criterion to a foodstuff as the primary means of control. In 1970, Sir Grahame Wilson, when summarizing a meeting on the use of microbiological criteria, stated: "Bacteriologists are better employed in devising means to prevent or overcome contamination than in examining more and more samples. Processing concerns the whole volume of food, sampling only a minute fraction of it."[79]

The pros and cons of relying on microbiological criteria to ensure microbiological safety will be addressed in the remainder of this chapter. For further consideration of management of microbiological hazards based on the use of Process and Performance Criteria, see ICMSF (1998).[37]

65.3.2 Definitions of Microbiological Criteria

Many definitions have been proposed for microbiological criteria.[35,56] The most recent of these, proposed in the Codex Alimentarius Commission's document on "Principles for the Establishment and Application of Microbiological Criteria for Foods,"[18,25] states that "A microbiological criterion defines the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot." This document separates such criteria according to their application by regulatory authorities and by industry but does not specifically name them according to usage; however, it is convenient to do so, and three types of microbiological criteria are generally recognized according to their use.[56] These are:

- **Microbiological Standard.** A mandatory criterion that is part of a law or ordinance.
- **Microbiological Specification.** Part of a purchasing agreement between a buyer and vendor of a food; such a criterion may be mandatory or advisory according to its use.
- **Microbiological Guideline.** A criterion used by a food producer to monitor microbiological conditions during the production of a food; such a criterion would be advisory and intended for internal use. Such a criterion could also be used as part of the verification process of HACCP.

65.3.3 Establishment of Microbiological Criteria

65.3.3.1 Need and Applicability

When considering establishing a microbiological criterion for a foodstuff or a range of foods, the first consideration should be that of need (eg, is there a health hazard associated with the food(s)?). The second equally important question that should be asked is: Will the criterion serve a useful function? For instance, a regulatory authority should consider whether the introduction of a criterion would have a positive health benefit (ie, reduce the risk of foodborne illness). A food producer may also wish to consider whether the application of a microbiological criterion would reduce the risk of spoilage. A third question that should be asked concerning application is: Is a microbiological criterion the best, or only, means of ensuring that a foodstuff is microbiologically safe or stable?

A regulatory authority wishing to establish a microbiological standard for a food should consider such need on evidence of a health concern derived from a full risk assessment or sound evidence of a public health concern.[18] Options for control should be considered as part of the process of risk management. One option may be in the form of a Food Safety Objective.[22] This has been defined by the ICMSF as "a statement of the frequency or maximum level of a microbiological hazard considered acceptable for consumer protection."[37] A microbiological criterion may be derived from a Food Safety Objective.

A food producer wishing to determine need should consider what public health concerns have been associated with the product or raw material(s) used, and is there microbiological or trade data indicating a significant spoilage problem with the intended product? He or she may also consider with respect to need:

- What is the potential for presence of pathogenic microorganisms in the raw materials?
- What is the effect of any processing on these (ie, will they survive or grow)?
- What is the potential for cross-contamination and growth of pathogenic microorganisms in the product?
- How will the product be distributed and how will it be used?

These questions are discussed in detail in Section 65.3.3.

Having established that there are one or more significant microbiological hazards associated with a foodstuff, the food producer must consider options for control. The questions that should be asked are:

- What numbers of microorganisms/amounts of toxins need to be controlled? Acceptable limits may be derived from legal limits or from recommendations of recognized bodies such as the ICMSF or the US National Advisory Committee on Microbiological Criteria for Food.
- With what degree of certainty should this control be exercised?
- What options are available for their control and how effective are these likely to be?
- Where should and can these control options be applied?

Consideration of the answers to these questions will help to clarify the need, content, and role of a criterion. For instance, it might be concluded that a microbiological safety or shelf-life concern is best controlled by designing or modifying a production process to eliminate a hazard(s) or to control it to an acceptable level, which, if successful, may mean that the use of a microbiological criterion as a means of control is not necessary, and a more effective means of control may be applied, such as the use of HACCP with control measures based on Performance and Process Criteria.[37]

As an example of the sort of considerations that should apply when considering options for the microbiological control of a product, we will consider the establishment of control measures for mayonnaise manufacture. The microbiological hazards and risks arising from the egg used in such a product could be avoided by the use of pasteurized egg purchased from an audited and approved source applying GMP and HACCP, or by purchasing egg from a less secure (and perhaps cheaper) source and using a microbiological criterion to clear the egg prior to use. However, the second option is likely to provide much less security than the first (because of poor assurance of detecting contaminated lots by microbiological testing) (Table 65–4). It may be decided, therefore, not to test, but to either reconsider the use of pasteurized egg or to reformulate the mayonnaise so that any pathogens of concern (eg, salmonellas) would be destroyed in the formulated product.

Consideration as to whether to apply a microbiological criterion as a control measure is never wasted effort because it is only by considering such options that the real need to use a microbiological criterion as a primary means of control will be identified. More often than not, it will be found that use of a microbiological criterion is the least-preferred control option, and should only be used when other more effective options cannot be applied or are found to be unreliable. Thus, the Codex Alimentarius document on microbiological criteria for foods[18] states, when referring to the application of such criteria, that: "They may be relevant to the examina-

Table 65–4 Probability of Acceptance of Food Lots with Different Levels of Contamination Using Two-Class Attribute Plans*

	% Chance of Accepting Lot with:				
Level of Contamination	*n* = 5	*10*	*15*	*30*	*60*
0.1%	99	99	98	97	94
1.0%	95	90	86	75	50
5.0%	78	60	50	25	5
10.0%	59	35	20	5	<1

*$n = 5$ to 60; $c = 0$; $m = 0$

Source: Adapted with permission from ICMSF, *Microorganisms in Foods 2,* Sampling for Microbiological Analysis: Principles and Specific Applications, 2nd ed., © 1986. International Commission on Microbiological Specifications for Foods.

tion of foods, including raw materials and ingredients, of unknown or uncertain origin or when other means of verifying the efficacy of HACCP-based systems and Good Hygienic Practices are not available." Potential uses and misuses of microbiological criteria by the regulatory authorities and by industry are considered in Section 65.3.4.

65.3.3.2 Basis

The basis of establishing a microbiological criterion has been well defined as a result of the combined activities of the ICMSF, FAO/WHO, and the Codex Alimentarius Food Hygiene Committee.[17,18,35] The following list of considerations that should be applied when establishing a microbiological criterion is based on the Codex document.[18] It assumes that the need to establish a criterion for a foodstuff has been established on the basis of the considerations discussed in Section 65.3.3.1.

The Evidence of Actual or Potential Hazards to Health. Although the Codex document[18] only concerns health considerations, spoilage and shelf-life concerns also may be considered for criteria applied by industry.

The Microbiological Status of Raw Material(s). This concerns the quality and type of raw materials used. The numbers and varieties of microorganisms found in raw materials will depend on a number of factors, including the source of the raw material, how it has been produced, and how it has been handled or treated since harvesting or production. Thus, if a raw agricultural product is not subject to any form of processing that might be expected to kill part or all of its microbial flora, the organisms present on that raw material will be more numerous and diverse than if the same raw material were pretreated before use. Thus, the microbial profile of a raw spice would be expected to be very different from that of an irradiated or heat-treated spice; such differences would need to be reflected in any microbiological criterion applied to the raw material or to a product containing this material. Clearly, if such spices were to be used in a ready-to-eat product, the criterion would need to include strict limits for infectious pathogens such as salmonellas. However, because of the poor security obtained by testing a product, even when the criterion requires as many as 60 sample units to be analyzed (see Table 65–4), the producer would be well advised to use only treated spices from an audited and approved source.

The Effect of Processing on the Microbiological Status of the Food. A food product made entirely of raw components will have a microbial flora that reflects the microbiological status of the individual components. Thus, a raw meat product would be expected, from time to time, to be contaminated with low numbers of zoonotic microorganisms such as salmonellas and campylobacters. However desirable it might be to totally avoid the presence of such organisms, it is unrealistic to expect this; any microbiological criterion needs to take this into account. On the other hand, a food made from cooked meat components would be expected to be free from such organisms and to contain fewer other microorganisms; a microbiological criterion should reflect this.

The Likelihood and Consequences of Microbial Contamination and/or Growth during Subsequent Handling, Storage, and Use. One of the most difficult aspects of setting a microbiological criterion is the need to understand sufficiently how any raw food materials and the intermediate and final product may be further contaminated or recontaminated during production, storage, distribution, and preparation for use, and the subsequent fate of such contaminants. Thus the microbial profile of a food product based on the sole consideration of microbes introduced with raw materials and the subsequent effect of processing may give a very inaccurate picture of true microbial profile of the final product. A well-founded HACCP study may provide some additional information, at least concerning potential routes and sources of contamination. Predictive models of microbial growth and survival also will provide useful information on the fate of microorganisms of concern in a food product, but accurate information concerning how a product is handled once it leaves the production site is seldom available and requires assumptions to be made of the likely handling conditions. Figure 65–3 indicates how a decision tree approach may be used to identify the organisms of concern in a chilled food, taking into account the factors mentioned above.

Category(s) of Consumer Concerns. As already discussed in Section 65.2.3.2, the possibility of foodborne illness resulting from the consumption of a contaminated food depends on a multiplicity of factors, including the inherent sensitivity of the consumer. Thus, a food intended for an infant should have a much tighter microbiological criterion than a food intended for an adult. Such a distinction between foods for infants and foods for adults is fine when a food is clearly labeled as infant food, but as many manufacturers have found to their cost, foods intended for consumption by an adult or young person may well be fed to an infant by a busy young mother, resulting in illness among the infants but not among adults consuming the same product. Thus, it is sound practice for a producer to label a food intended for an adult population, if it is not suitable for an infant or other sensitive sector of the population. Examples of such foods are honey, as there is evidence of infant botulism from its consumption (see Chapter 41), and certain types of soft cheeses that should not be consumed by women when pregnant because of the increased risk of listeriosis (see Chapter 44).

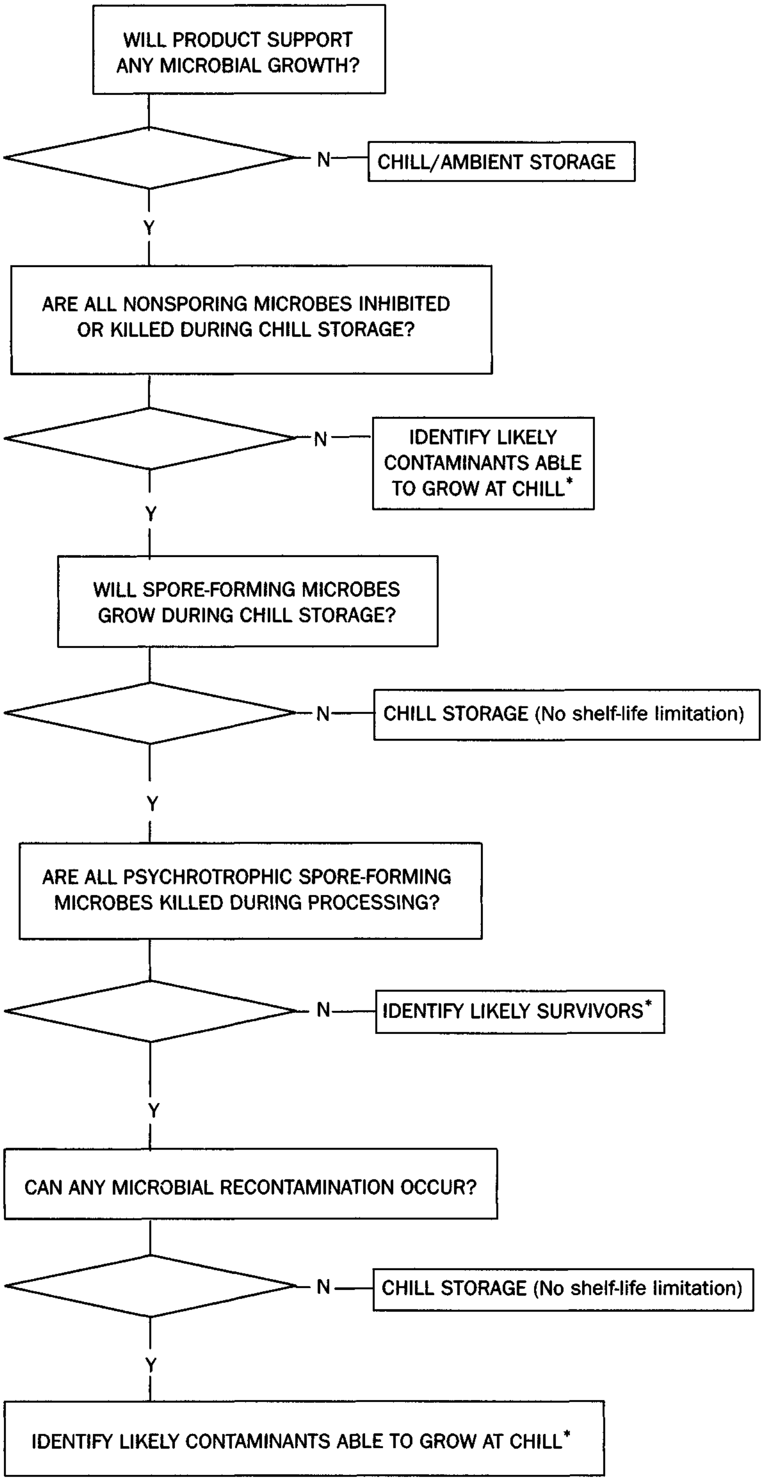
The Cost/Benefit Ratio Associated with the Application of the Criterion. The costs involved in applying a microbiological criterion can be substantial. These include the cost of sampling (manpower and food costs) and performing the microbiological analyses. They may also include costs of transporting the samples to an offsite laboratory for testing; communicating the results of the analysis accurately and in a timely manner so that product can be released or withheld; and the cost of actions arising if a product does not meet the criterion. Such actions may include sorting, reprocessing, or destruction. Usually, substantial costs will be involved in the recovery of product from distribution sites or the marketplace if failure to meet a criterion is identified after a product has entered the distribution chain. Producers who elect to hold product until results of microbiological analysis are available must build this into their inventory control system along with the cost of storage onsite or at an offsite location. This may necessitate double handling at a warehouse or distribution center. For perishable foods, the time the product is held awaiting analytical results is loss of shelf-life time, and this can decrease the geographical distribution area for highly perishable foods. Similar considerations apply when product is held for analysis by an authority at a point-of-entry into a country.

Further costs may arise from the statistics of the sampling plans as these have a defined probability of failure. Such failure may result in faulty product meeting the sampling plan and is called the consumer's risk and is defined by the ICMSF[35] as "the probability that a lot will be accepted on the basis of results of testing analytical samples when in reality the lot as a whole is substandard relative to the stated criteria of acceptability." Failure to meet a sampling plan also may occur with a completely acceptable product and is called the producer's risk and is defined by the ICMSF[35] as "the probability that a lot of acceptable quality will be rejected on the basis of results of testing analytical samples relative to the stated criteria." The statistics of sampling plans, including producer and consumer risk, are discussed in Chapter 60.

It cannot be overemphasized that sole reliance on a microbiological criterion as a means of identifying defective product is likely to have high cost and low benefit. The use of a microbiological criterion as part a quality assurance function based on GMP and HACCP is likely to have a much better cost/benefit ratio and will be considered in Section 65.3.4.2.

65.3.3.3 Components

A microbiological criterion, irrespective of how it will be used, should comprise basic components.[18,25]


WILL PRODUCT SUPPORT ANY MICROBIAL GROWTH?
N
CHILL/AMBIENT STORAGE
Y
ARE ALL NONSPORING MICROBES INHIBITED OR KILLED DURING CHILL STORAGE?
N
IDENTIFY LIKELY CONTAMINANTS ABLE TO GROW AT CHILL*
Y
WILL SPORE-FORMING MICROBES GROW DURING CHILL STORAGE?
N
CHILL STORAGE (No shelf-life limitation)
Y
ARE ALL PSYCHROTROPHIC SPORE-FORMING MICROBES KILLED DURING PROCESSING?
N
IDENTIFY LIKELY SURVIVORS*
Y
CAN ANY MICROBIAL RECONTAMINATION OCCUR?
N
CHILL STORAGE (No shelf-life limitation)
Y
IDENTIFY LIKELY CONTAMINANTS ABLE TO GROW AT CHILL*

*These microbial contaminants are potential microbial hazards and their significance as hazards in a particular chilled food manufacturing operation should be determined by the application of HACCP and options for control (including the use of microbiological criteria) established by this process.

Figure 65–3 Identification of Microbes of Concern in Chill-Stored Food

A Statement of Microorganisms of Concern and/or Their Toxins/Metabolites and the Reason for That Concern. All microbiological agents of potential concern should be considered, including bacteria, fungi, viruses, parasitic protozoa, and toxic algae, together with their spoilage and toxic metabolites such as mycotoxins, pressor amines, and the microbially derived marine toxins. The reasons for inclusion of an agent in a criterion should be given and whether it is included because it is a spoilage organism, shelf-life indicator, hygiene indicator, or direct health concern. Only organisms of direct relevance to the safety or keepability of a particular foodstuff should be included in a criterion. General statements such as "pathogenic microorganisms absent" should not be used.

The Analytical Methods for Their Detection and/or Quantification. There are a number of considerations when selecting analytical methods for use when applying a microbiological criterion.

The first is the reliability of the analytical procedure (ie, its accuracy and repeatability). There have been many studies on the reliability of microbiological methods and their dependency on:

- the counting procedure used—methods based on standard three-tube most probable number techniques have an accuracy of c. +/- 400% (95% confidence interval)[12] whereas direct count techniques have an accuracy of c. +/- 50% for counts of between 30 and 200 CFUs, this dropping to an accuracy of c. +/- 150% for counts below 10 CFUs (95% confidence interval)[11]
- interference by food substrates, sanitizers, and other microorganisms—many foods and food ingredients are markedly inhibitory to some microorganisms (eg, chocolate and spices), and these will particularly affect the detectability of microorganisms in presence/absence tests; the presence of sanitizers such as quaternary ammonium compounds also may reduce detectability of organisms on food processing equipment. Also, the presence of large numbers of other organisms acting either as competitors to growth or making conditions in the growth medium unsuitable for growth can reduce the detectability of an organism
- the degree of injury of the target microorganisms—selective microbiological methods used for counting or detecting specific microorganisms will perform very unreliably if organisms are damaged as a result of exposure to harmful agents such as heat or acid.

Many of the factors affecting the detection or countability of microorganisms can be reduced or eliminated by pretreatment of the material to be analyzed and selection of the method of analysis. The procedures for doing this are discussed in Chapter 62.

The second consideration is the intended use of the method. Thus, if the food is to be examined for compliance with a legal standard, the method used should be that specified in the standard; such a method should be one that has been approved as a reference method by an international standards organization such as ISO,* AOAC,* or CEN,* and should be shown to perform satisfactorily with the foodstuff(s) to which the criterion applies. If such a method does not exist for a particular agent, that agent should not be included in a legally binding microbiological criterion. If the method is to be used as part of a criterion intended for use by industry for internal control purposes only, then a less proven method may be acceptable, particularly if it enables more samples to be examined, and the results to be obtained faster and/or at a reduced cost. In this regard, some of the newer rapid methods may be more applicable (see Chapter 61).

A Plan Defining the Number of Field Samples To Be Taken and the Size of the Analytical Unit. The keystone of any microbiological criterion is the sampling plan, as the operating characteristics of this define the degree of confidence whereby a particular concentration of a microorganism or toxin can be measured or detected.

Sampling plans, as they may be used by industry and public health authorities for the purpose of food lot acceptance, will be mentioned briefly. For detailed information on the statistics of these, and sampling plans used for investigation, survey, or other purposes, the reader should refer to Chapters 58 and 60.

Two types of sampling plan are used for acceptance sampling of food lots: attribute plans and variable plans.

1. Attribute Plans. An Attribute Plan is defined by the ICMSF:[35] "A sampling plan in which each selected sample unit is classified according to the quality characteristics of the product and in which there are only two or three grades of quality; for example, acceptable, defective; absent, present (these are Two-Class Attribute Plans); acceptable, marginally

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*ISO, International Organization for Standardization; *AOAC*, Association of Official Analytical Chemists; *CEN*, Committee European Normalization.

acceptable, defective; low count, medium count and high count (these are Three-Class Attribute Plans)". Two-Class plans are mainly used for foodborne infectious pathogens, and Three-Class plans for general, indicator, and some toxigenic microorganisms.

Two Class Attribute Plans have a single limit (eg, a microbial concentration) that separates one attribute (eg, an acceptable concentration of a microorganism) from a second attribute (eg, an unacceptable concentration of microorganisms). The decision-making part of such a plan is defined by three letters. The first is represented by the letter n, which is the number of sample units taken from a food lot to be tested; the second is represented by the letter c and is the maximum number of sample units allowed to have concentrations of microorganisms above the acceptable level before the lot of food under test is judged to be defective; the third number is represented by the letter m, which separates an acceptable from an unacceptable concentration of microorganisms.

Thus, a Two-Class Attribute sampling plan for salmonella in a cooked meat product may state: n = 5; c = 0; m = 0; analytical unit (amount of Sample Unit analyzed) 25g. This means that five Sample Units should be taken at random from a food lot and from each of these, 25g taken for analysis. If salmonellas are detected in any of the Sample Units analyzed, the lot fails the criterion.

The difference between a Two-Class and a Three-Class Attribute Plan is that instead of two attribute classes (acceptable and unacceptable), there are three; the third being "marginally acceptable." As the name implies, the marginally acceptable attribute separates the acceptable from the unacceptable. The letter n is used to define the number of sample units to be analyzed from a food lot, but c in a Three-Class plan is the maximum number of marginally acceptable Sample Units acceptable before a food lot is judged to be defective. The m value in a three-class plan becomes the value that separates an acceptable from marginally acceptable concentration of a microorganism or a toxin. A new letter, M, is introduced to separate a marginally acceptable concentration from an unacceptable concentration of an organism or toxin.

There are two particular attributes of Three-Class plans that are not well recognized. The first is that the performance of a Three-Class plan is affected markedly by the distribution of microorganisms in the food lot examined.[35] The second is that there is no fixed relationship between m and M, as the former is set by the performance of a food production plant when operating according to GMP and the latter is based on an expert opinion of the maximum concentration of a pathogen, toxin, or spoilage organism that is considered acceptable in a food, taking into account how that food may be handled and consumed and allowing a certain safety margin. Thus, M is based on a risk assessment and m, by what is achievable by a production plant operating under accepted conditions of GMP. The m value normally would be expected to be at least 10 times less than the M value.

For example, a Three-Class Attribute Plan for *Staphylococcus aureus* in the lot of a cooked meat product may state: n = 5; c = 2; m = 1,000 g^{-1}; M = 10,000 g^{-1}. This means that the cooked meat lot would be acceptable, if three or more of the five analytical units had concentrations of *S. aureus* of equal or less than 1,000 g^{-1} and two or less with concentrations of microorganisms between 1,001 g^{-1} and 10,000 g^{-1} and no analytical unit with a concentration of microorganisms above 10,000 g^{-1}. Three or more analytical units with concentrations of *S. aureus* above 1,000 g^{-1}, and any above 10,000 g^{-1}, would result in the lot being judged to be defective.

2. Variables Plans. The ICMSF[35] describes a Variables Plan as a sampling plan in which the criteria employ the statistics of the sample, such as the mean and standard deviation, rather than the segregation of sample units into attribute classes according to their microbial content as is done when using an Attribute Plan. Such measurement provides the possibility of using a sampling plan with better discrimination between acceptable and defective product lots than is achievable using an Attribute Plan.[43] Their better use is based on the fact that if the distribution of microorganisms in foodstuffs approximates to log normality, the statistics developed for log normal distributions can be applied. However, this limits their use to situations where microbial distributions can be measured; thus, they mainly are used for in-plant testing. For further information on the use of Variables Plans, see Chapter 60.

Microbiological Limits Considered Appropriate to the Food at Specified Point(s) in the Food Chain. For each microorganism or toxin of concern in a foodstuff, one or more limits must be ascribed in the sampling plan. Thus, as noted above, the m value in a Three-Class Attribute Plan should be based on what is achievable in a food operation complying with accepted GMP, whereas the M value in such a plan is a limit corresponding to an unacceptable or hazardous level of contamination. The m value in a

Two-Class Attribute Plan similarly specifies the limit of acceptability.

The *m* limit values in Two-Class Plans and the *M* limits in Three-Class Plans should ideally be derived from some formal risk assessment procedure, such as described in Section 65.2, or when the results of such an assessment are not available, which will usually be the case, on the consensus views of food microbiologists knowledgable of the microbiology of a foodstuff and the significance of particular microorganism(s) of concern. The ICMSF, in its book on microbiological criteria,[35] gives guidance on the choice of limits for *m* and *M* values and provides examples of their application in sampling plans for different types and classes of foods. The limits in these plans are based on the views of food microbiologists from different countries who are expert in the foods and microorganisms of concern. They provide useful guidance for establishing microbiological criteria.

Point in the Food Chain Where the Criterion Applies. The place where a microbiological criterion is to apply must always be specified, as microbial numbers and risk change as a food proceeds along the food supply chain to the consumer. Thus, a food capable of supporting the growth of a particular foodborne pathogen identified in a criterion, and that is likely to be stored at temperatures at which growth could occur, would need to meet a tighter limit when tested at a point-of-entry to a country, and to meet an even tighter limit if tested immediately after production. For example, the ICMSF has recommended that the maximum concentration of *L. monocytogenes* that is acceptable in a ready-to-eat food is 100 g^{-1};[33] this limit is the acceptable concentration at the point of consumption and can be considered to be a food safety objective.[37] Thus, in order that this level is not exceeded in a product supporting the growth of this organism (such as a chilled stored cooked meat product), the concentration acceptable immediately following production will be considerably less than this and, for extended shelf-life products, may require that the production operation is designed and operated so that the organism will be absent and any microbiological criterion should reflect this. The use of mathematical models predicting microbial growth under different storage conditions can provide useful guidance in estimating such changes; see Chapter 16.

There may well be sound arguments that a microbiological criterion is best applied to a food at the point-of-sale, as this would better reflect the microbiological status of the food when offered for sale. However, difficulties arise when food is found to be defective at this late stage in the food chain. For example, some of the product is likely to have been purchased or even consumed. Furthermore it may be difficult to identify where to place the fault (or responsibility) that led to the food failing to meet the criterion. Dispersion of the food lot to many different retailers or food service establishments could make the recovery of a defective lot difficult, if not impossible. Also, although product sampled at one sales point may fail to meet a criterion, this does not necessarily mean that all food from the same food lot is defective; for instance a chilled food lot may be distributed to a number of outlets and due to a failure to control the temperature of the food in one of these outlets, or its distribution chain, the product failed the criterion, yet all other food comprising the lot was correctly handled and therefore not defective. Further, as foods move along the food chain, the discrete identity of production lots is likely to be lost and testing may have to be done on mixed lots, which is likely to further reduce the discriminatory power of a sampling plan as microbial distributions will tend to be more heterogeneous in mixed lots; this may be a particular problem when sampling food in international trade as consignments may contain several food lots.

If samples of food collected at retail are taken for microbiological analysis, the purpose of such sampling should be clear (eg, is this to check whether the production process, or subsequent handling, has been satisfactory, is it part of a microbiological survey to assess the general status of foods in the market, or is it to determine whether a food is safe to eat?). The dynamic nature of microbial populations in many foods needs to recognized when such analyses are assessed. Not only is there likely to be an increase in the total population of microorganisms in perishable foods from the time of production to use-by date, but there also is likely to be a change in the relative proportions of the different organisms making up this population. Thus, it is important that the point in the food chain where a microbiological criterion is to apply is clearly specified and a criterion intended to be applied at one point in the food chain is not applied at another.

Number of Analytical Units That Should Conform to These Limits. The number of Sample Units that should be analyzed for compliance with specified limits will always be a balance between what is desirable to provide confidence that a particular level of contamination is unlikely to be exceeded, and the number that it is feasible to test.

An example of the interrelationship between sample units tested and probability of detection of different levels of contamination in a food lot using Two-Class Attribute Plans (where $c = 0$ and $m = 0$) is presented in Table 65–4.

Clearly, the more sample units that can be tested, the greater the chance of detecting a food lot contaminated, for example, with low levels of salmonellas. Thus, if 1% of units in a food lot were contaminated with salmonellas, and five Sample Units were randomly taken from this food lot and analyzed, there would only be a 5% chance of detecting the salmonellas and rejecting the lot; if 60 Sample Units were examined, the chance of detecting salmonellas would increase to 50%. However, even when 60 Sample Units are analyzed, there is still only a one chance in two of the lot being found contaminated and being rejected. Thus, the limitations of microbiological testing must always be recognized and such limitations taken into account when applying microbiological criteria; no amount of testing can ensure complete safety.

The number of Sample Units specified in a mandatory microbiological criterion (and where specified, the size of the analytical unit) should be adhered to, and not modified, as this will change the performance characteristics of the sampling plan.[18,35] However, in some circumstances, the stringency of a sampling plan used for internal control by industry may need to be tightened; this is usually achieved by increasing the number of Sample Units tested. Circumstances where this may be necessary include where there is no, or only limited, knowledge of the ability of a new supplier of a raw material to meet the microbiological requirements for its use, or when an existing supplier is found to be failing to meet an established criterion. They may also include new production, or a decision by marketing to target a product at a particularly sensitive sector of a population such as young children.

Action To Be Taken When a Criterion Is Not Met. If when tested, a food lot does not comply with the defined criterion of acceptance (as specified in the microbiological criterion), the food should not be repeat tested on the basis that it has a chance of passing the next time. If a product passes a criterion, whereas other information suggests that it should fail, the use of an investigational sampling plan would be more appropriate (see Chapter 60).

A food lot failing to comply with a mandatory microbiological criterion must be rejected for its intended use; however, rejection should not necessarily lead to destruction. An assessment should be made as to whether the defective lot can be sorted, reprocessed, or used for another purpose.[18] Such an assessment should be done only in consultation with people who are knowledgeable of the microbiological risks associated with the defective lot. Additional testing may be appropriate before finally deciding on the disposition of the lot. For example, it may be possible to divide a production lot into sublots according to the time of production and to sample the sublots. One means of doing this is to sample production from each consecutive pallet of a production lot to determine if there is a pattern to the contamination. For instance, the first 4 hours of a production run may be unacceptable due to startup problems. Thus the additional testing may indicate that product from 6 AM to 10 AM is unacceptable. A decision could then be made to reprocess all the product from 6 AM to 12 PM or 2 PM, acknowledging that the sampling plan would be unlikely to detect low levels of defective product. Such a decision should always reflect the nature of the defect and the risk to the consumer of releasing defective product. Failure to meet a criterion used as part of the process of verification of HACCP will require investigation of the process to establish where the failure occurred that led to the defective food lot. This may necessitate modification and revalidation of a CCP.

65.3.4 Uses of Microbiological Criteria

The uses of microbiological criteria are discussed in the following paragraphs. They can be separated broadly into their use by the regulatory authorities, mainly as legal requirements, and their use by industry to meet legal requirements and for internal control purposes. Their uses are listed in Table 65–5.

65.3.4.1 Application by a Regulatory Authority

The main use of a microbiological criterion by a regulatory authority is as a measure of checking that a food offered for sale is likely to be safe to eat. Although regulations will be mainly in the form of mandatory criteria, and will generally apply to product after manufacture, they may apply specifically to product during processing and may not always be mandatory. Four examples, showing the range of usage of microbiological criteria by several different regulatory authorities, are presented and discussed below.

Table 65–5 Uses of Microbiological Criteria

Regulatory Use	*Industry Use*
• Regulations:	• Compliance with regulations
Import control	• Purchasing agreements*
Domestic control	• Establishing an HACCP plan
• Monitoring industry	• Verification of HACCP plan
• Advising industry	• Product/process monitoring

*particularly microbiological specifications

The USDA, in response to outbreaks of *E. coli* O157:H7 associated with ground beef, declared *E. coli* O157:H7 an adulterant, and that ground beef found to contain this pathogen must be withheld, or if distributed, recalled from the market.[74] Because an entire production lot could be subject to recall, the definition of a lot is important. For this regulatory program, a lot was defined as the product produced between two complete cleaning and sanitizing procedures. Thus, if an operation is cleaned and sanitized every 4 hours, then the product produced during the 4-hour period in which the sample was collected would be subject to recall if the sample tested positive. In addition, if the raw ground beef is used as an ingredient in other raw products, these secondary products would be subject to recall. The program involves collecting samples from the grinder as the meat is ground; this is the best available sampling point as the distribution of microorganisms in the meat will be most homogeneous at this stage in the process, and hence *E. coli* O157:H7, if present, most detectable.[42] The samples are collected in federal- and state-inspected establishments, as well as in retail stores where beef is ground for local sale. A single portion of ground beef is collected from the lot. The program has involved application of a sampling plan consisting of $n = 1$; $c = 0$; $m = 0$; the single Analytical Unit is 25g.

During the fiscal period 1995 through 1997, the USDA found 9 lots to be positive out of about 16,500 lots sampled, giving a prevalence rate of approximately 0.05%. Assuming that the contamination of *E. coli* O157:H7 is randomly distributed throughout the ground beef supply, it can be estimated that examination of 600 Sample Units would be necessary for a 95% probability of detecting a contaminated lot, if the level of contamination in the lot is 0.5%. This probability would decrease to 45% if the level of contamination is 0.1%. Thus, since the accumulated data for ground beef have shown a level of contamination of 0.05%, it is evident that a lot acceptance plan based on testing even 100 Sample Units from a lot would have little chance of detecting a defective lot and thus would not alter the risk of distributing contaminated ground beef. This is illustrative of the impossibility of detecting a low level of a highly infectious organism such as *E. coli* O157:H7 in a foodstuff. At best, the USDA regulation sends a message to the meat and retail sectors of the food industry that this pathogen is unique in its public health impact and its presence in ground beef must be prevented to the extent possible.

The second example of the application of a microbiological criterion by a regulatory authority is taken from the European Union Directive on Milk and Milk-based products (EC Directive 92/46).[19] This directive contains a number of microbiological criteria including the criteria for drinking milk, which are reproduced below.

- Raw cow's milk for drinking must meet the following standards after bottling/packaging:
  —Plate count at 30° C (per ml): = or <50,000 (geometric average over a period of 2 months, with at least two samples/month);
  —*Staphylococcus aureus* (per ml): $n = 5$; $c = 2$; $m = 100$; $M = 500$;
  —Salmonella: $n = 5$; $c = 0$; $m = 0$ (analytical unit, 25g);
  —In addition, pathogenic microorganisms and their toxins must not be present in quantities such as to affect the health of consumers.
- In random sampling checks carried out in the treatment establishment, pasteurized milk must meet the following microbiological standards:
  —Pathogenic microorganisms: $n = 5$; $c = 0$; $m = 0$ (analytical unit, 25g);
  —Coliforms (per ml): $n = 5$, $c = 1$, $m = o$, $M = 5$;
  —After incubation at 6° C for 5 days, plate count at 21° C (per ml): $n = 5$, $c = 1$, $m = 50{,}000$, $M = 500{,}000$
- In the random sampling checks carried out in the treatment establishment, sterilized milk and ultra-high temperature (UHT) milk must meet the following standard after incubation at 30° C for 15 days:
  —Plate count (30° C): = or < 10 (per 0.1 ml)
- When the maximum standards and compulsory criteria are exceeded and when subsequent investigation indicates a potential danger to health, the competent authority shall take appropriate measures.

How well does this legislation meet the principles proposed for setting microbiological criteria by the Codex Alimentarius Food Hygiene Committee? The points it does cover in relation to the Codex Principles are: it clearly tells the user what foods the criteria apply to and where the criteria are to be applied; it uses Two- and Three-Class Attribute sampling plans and provides some guidance on frequency of testing; it also informs the milk processor what needs to be done when a product fails to meet the acceptance criteria. While the Directive does not give methods of analysis (except where they are specifically required for a product), the UK Dairy Product (Hygiene) Regulations (1995) that are based on this Directive contain a schedule of methods of analysis. Analyses, when done for legal purposes, would be done in an European Union (EU) reference laboratory. Such a laboratory would be operating to appropriate CEN standards, accredited by an approved body, and would use approved methodology. What the Directive does not do is to always provide information of pathogens of concern. Thus it states "pathogens" absent in 25g; "other pathogens" absent in 25g; and "Pathogenic microorganisms and their toxins must not be present in quantities such as to affect the health of the consumer." Such catch-all requirements should not be included in regulations, and we are pleased to note that they are absent from the UK Regulation based on this Directive.

The Directive also makes use of the concept of mandatory and advisory criteria. Thus, it specifies mandatory criteria for salmonellas and *L. monocytogenes* in milk products requiring product to be withdrawn if these "standards" are exceeded. It then lists a series of sampling plans for organisms "indicative of poor hygiene" (*S. aureus* and *E. coli*) requiring a review of "the implementation of the methods for monitoring and checking critical control points." This is an example of the use of criteria for HACCP verification and requires the authorities to be informed of what corrective procedures have been introduced to prevent the reoccurrence of the fault that led to a count limit being exceeded. It further requires that if specified products are found to contain concentrations of the designated microorganisms above *M*, they should be tested for toxicity (eg, enterotoxin production by *S. aureus*) or pathogenicity (eg, *E. coli* O157:H7) and if these are found to be toxigenic or pathogenic, the product must be withdrawn from sale.

The Directive also has a third category of criteria, "Guidelines," giving sampling plans and limits for "indicator organisms." The Directive states that "These guidelines should help producers in ensuring proper operation of their establishments and in carrying out their own checks on their production."

Thus, this Directive very much follows the spirit of the Codex principles for establishing microbiological criteria. It defines mandatory requirements for pathogens in specified products; the use of organisms indicative of poor hygiene control for the checking of GMP and HACCP; and the use of further organisms (coliforms and "plate count") in "guidelines" to be used solely by manufacturers. This is a good example of the application of the Codex Principles in a regulatory framework.

A third example of the application of microbiological criteria is their use by the Canadian authorities for *L. monocytogenes* control.[26,45] The Canadian policy for this organism incorporates the inspector's opinion of whether a food production plant is applying adequate GMP to control the potential for product contamination. If the inspector concludes that adequate GMPs are not being applied, then environmental samples are collected to assess the probability of finished product being contaminated with pathogens, including *L. monocytogenes*. On the basis of the environmental sample results, the next step may be to sample the finished product. The sampling plan applied is shown in Table 65–6.

This is a sound and measured approach to food control based first on inspection and assessment of GMP, and second, where appropriate, by applying microbiological criteria, based on an estimate of consumer risk.

The fourth example is the USDA Pathogen Reduction: HACCP regulation[73] wherein regulatory performance standards were established for salmonellas on carcasses and in raw ground meat and poultry. The criteria were based on data obtained through nationwide baseline studies and involved a statistical procedure called "the moving sum." This procedure compares the number of positive samples in a series of consecutive samples (called a "window") to an acceptable limit. The frequency of sampling (eg, 1/day, 5/week, or 1/week) is not stated in the regulation but will vary according to a plant's performance (ie, plants having difficulty in meeting the standard would be sampled more frequently by the agency). The number of samples tested (n) and the maximum number of positives permitted (c) were calculated for each meat and poultry category to provide approximately an 80% probability of passing when the plant is operating at the national baseline for the prevalence of salmonellas in the category of product tested (ie, just within the performance standard). Examples of performance stan-

Table 65–6 Canadian Government Sampling Plan for *Listeria monocytogenes* in Ready-to-Eat Food Products

Food Category	*Sampling Plan*
1. Foods that have been linked to listeriosis.	$n = 5$; $c = 0$; $m = 0$; analytical unit, 50g
2. All other RTE foods supporting *L. monocytogenes* growth and having a refrigerated shelf life of >10 days.	$n = 5$; $c = 0$; $m = 0$; analytical unit, 25g
3. RTE foods supporting *L. monocytogenes* and having a refrigerated shelf life of equal or <10 days and all foods not supporting growth.	$n = 5$; $c = o$; $m = 100\ g^{-1}$; analytical units, 5 x 10 g analyzed separately

RTE, ready-to-eat.

Source: Data from J.M. Farber and J. Harwig, The Canadian Position on *Listeria Monocytogenes* in Ready-to-Eat Foods, *Food Control*, Vol. 7, pp. 253–258, © 1996, Butterworth-Heinemann, and A.M. Lammerding and J.M. Farber, The Status of *Listeria Monocytogenes* in the Canadian Food Industry, *Dairy, Food and Environmental Sanitation*, Vol. 14, pp. 146–150, © 1994, International Association of Milk, Food and Environmental Sanitarians, Inc. (IAMFES).

dards and sampling plans include: steers/heifers (performance standard, 1%) $n = 82$, $c = 1$; hogs (performance standard, 8.7%), $n = 55$, $c = 6$; and ground chicken (performance standard, 44.6%), $n = 53$, $c = 26$. The "window" for each plant will move chronologically as samples are collected and results become available for each category. Compliance will be based on the most recent series of samples. For example, for steers/heifers there can be no more than 1 positive sample among the last 82 samples tested. If a plant fails to meet a performance standard, it must take immediate action to comply with the standard by reviewing its procedures. If the plant fails to meet the required standard for a second consecutive series of samples, this will require the plant to review its HACCP plan and implement corrective actions. If it fails a third series, this could lead to withdrawal of federal inspectors until such time that the agency approves of written assurances that corrective actions will lead to compliance with the standard. This application of a microbiological criterion is intended to measure the performance of a plant's pathogen control measures over time by testing for a pathogen (salmonella). If the original baseline data are valid, then plants that have contamination levels in the top 20% will be forced to make corrections or will be closed. There are, however, a number of uncertainties associated with the designated performance standards, including failure to take account of seasonal and regional differences during the baseline studies. This regulation was only implemented in January 1998, and whether it will result in a reduction in disease from salmonella and other enteric pathogens has yet to be determined.

Summary. A mandatory microbiological criterion should only be applied by a regulatory authority when there is documented evidence that a food is likely to contain microorganisms or toxins that are a hazard to health and that other measures are likely to give less effective control. Legal requirements for limits for foodborne pathogens and toxins should be based on risk assessment. For reasons described and shown above, the sole reliance on a testing for compliance with a microbiological criterion can never provide proper consumer protection. The regulatory authorities should continue to encourage industry to apply GMP and HACCP as the primary means of microbiological control because this will give much greater assurance of microbiological safety than microbiological testing to detect contaminated product.

65.3.4.2 Application by Industry

Microbiological criteria may be used by industry for compliance with regulations, as part of a purchasing specification, and establishing and verification of HACCP. The procedures described here may also be followed by governmental agencies purchasing foods for the military, hospitals, welfare programs, schools, etc.

Compliance with Regulations. A producer may use the same microbiological criterion for internal control purposes as that required in a regulation. However, because of the heterogeneity of distributions of microorganisms and toxins in most foods, the variable performance of microbiological analytical methods, and the performance characteristics of sampling plans (eg, consumer's versus producer's risk), there is often a high probability of a food lot meeting a criterion when tested by a producer failing when tested by a regulatory agency. To reduce this probability, the producer can do a number of things including one or more of the following:

- examine more and/or larger Sample Units (where appropriate, these may be composited for testing) and/or apply tighter limits for food lot acceptability
- use an automated sampling device that takes small product samples frequently and regularly from a production line and composite these—this has been successfully used for salmonella testing in dried milk plants
- select a time or a point in the production process where a contaminant, if present, is most likely to be detected (eg, in food residues on equipment, in environmental samples, or where a contaminant is most likely to be most homogeneously distributed)
- use a variables-based sampling plan.

Purchasing Specification. Such specifications may be between a raw material or ingredient supplier and a food manufacturer, or between a manufacturer and a customer, such as a retailer. The content of a microbiological criterion for these two purposes will be broadly the same and will comprise identification of the foodstuff(s) to which the criterion applies (this should include an agreed definition of a food lot); a listing of organisms and/or toxins to which the foodstuff(s) should comply; the food lot acceptance sampling plan to be applied including limits for each microorganism, metabolite, or toxin; analytical technique to be used (sampling method, procedure for storage of samples, preparation of sample units for analysis and methods of analysis); point(s) of analysis, eg, before a particular processing step, after packaging; frequency of testing (every lot or specified skip lot testing); action(s) to be taken when lot does not meet the microbiological specification (including agreed method of reporting out-of-specification product lots). The testing done to ensure compliance with a buying specification may be done by a laboratory operated by the supplier, the purchaser, or a third party laboratory; it is most important that all concerned are aware of the sampling procedures, the methods of analysis, and the reporting procedures.

The extent to which a microbiological criterion will be applied as part of a purchasing specification should depend first on the microbiological concerns associated with the raw material or product and second, the demonstrable degree of production control exercised by the supplier. For instance, has the supplier's production unit been audited to establish that GMP and quality assurance systems are in place and whether a validated HACCP system is operational? Can control and monitoring records at CCPs be made available to the purchasing company? Third, what is the track record of the supplier? Product from a new supplier will initially require more frequent testing (eg, every food lot) than an established supplier with a good record of compliance and particularly where the supplier's CCP monitoring and verification data are available to the purchaser. If product is to be purchased from a factory where the purchaser has limited or no knowledge of the control procedures that are in place, for instance, because it is located in a relatively inaccessible part of the world, some knowledge can be obtained by asking a supplier to complete a questionnaire asking for details of production methods and control procedures (including management systems). However, information obtained in this way often is incomplete or questionable, so the buyer may have to rely on the use of a lot acceptance sampling plan to check that the product meets the buying specification. In this situation, the purchaser may apply a system of hold and test (the product is not used or dispatched until the results of analysis are available) and apply a more extensive range of tests and/or stricter limits to a lot to provide additional assurance of microbiological acceptability.

A buying specification applied by industry should never form the basis for a legally enforceable microbiological criterion for the following reasons.

- The types of organisms included in a buying specification may be because the food is to be used for a particular purpose (eg, thermophiles in raw materials used in canned foods to be distributed in tropical countries).
- The limits applied in a buying specification will usually be much stricter than those applied in a regulation for a number of reasons (see above), including the need for low count raw materials for an extended shelf life of chill-stored product. Thus, a very tight limit for *L. monocytogenes* may be appropriate in a sampling plan applied to a chill-stored, ready-to-eat product by a purchaser such as a retail store; such a limit would be much tighter than that used in a regulation so that the level of listerias in the product at the end of shelf life does not exceed a safe level.
- The level of sampling applied to a product that is tested on a regular basis in a production plant operating to GMP and applying HACCP may not be the level of sampling that would be appropriate for a food lot, when examined by a third party with no knowledge of the control measures applied during production.

Establishing and Verification of HACCP. Limits for microorganisms or toxins in microbiological criteria may have a useful role in HACCP.[56,65] They may be used in an HACCP study as an indication of the maximum concentrations acceptable in a foodstuff and thus may be used to identify a suitable critical limit for a CCP. However, absence of a limit for a particular pathogen in a recognized criterion should not imply that it should not be considered in an HACCP study as the identification of hazards should always be based on a hazard analysis of an actual food operation (see Chapter 58).

The process of "verification" mostly is defined in the context of its use in HACCP, but it can also apply to verification of GMP. The definition of verification applied by Codex[25] is: "The application of methods, procedures and tests, in addition to monitoring, to determine compliance with the HACCP plan." Verification is defined in this way in other documents on HACCP[39,52] in order to clarify that the process of verification is additional to the monitoring procedure routinely applied to ensure appropriate control at a CCP. Monitoring and control at a CCP are almost entirely achieved by applying nonmicrobiological methods because of the slow response time and poor accuracy of microbiological methods. The process of "validation" is applied to "obtaining evidence that the elements of the HACCP plan are effective."[25] Unlike verification, it is not an ongoing activity and is only intended to support the initial establishment of the HACCP plan.

Although one of the main uses of microbiological criteria within HACCP is in verification, lot acceptance testing, using Attribute or Variables Sampling Plans, may be used to monitor a particularly microbiologically sensitive raw material such as a spice for salmonella. For instance, if this were to be used as a flavoring agent in a ready-to-eat snack product, the spice would not be used until the results of an analysis indicated that it was negative for salmonella, as spices, depending on type, source, and treatment may be contaminated with salmonellas. Thus, testing the ingredient in this instance is an act of monitoring and the critical limit for the CCP (ie, the raw material) is defined as the microbiological criterion. Also, for some products, and particularly where the HACCP study identifies that a hazard may not be reliably controlled to an acceptable level, it may be appropriate to apply a microbiological criterion to an end product as part of production control. In this case, the product is held pending the results of the monitoring process that consists of applying an attribute or variables sampling plan to determine whether the product complies with the established criterion. It is important to recognize that in HACCP, monitoring is distinct from verification as the former is used to routinely monitor that control is achieved at a CCP, whereas verification is an additional procedure aimed at confirming that the HACCP plan provides the intended degree of control over microbiological and other hazards identified in the plan. Thus, verification may be applied to a newly established HACCP plan or to a plan that has been in operation for some time. Many procedures may be used in the process of verification (see Chapter 58), but we are only concerned here with the use of microbiological criteria for this purpose.

One of two strategies may be used if a microbiological criterion is to be used in the verification of HACCP. The first is to follow the advice in the ICMSF book on HACCP,[34] which is to apply a sampling plan that is much more rigorous and searching than that which might be applied in a routinely applied sampling plan, recognizing that it should test the performance of the HACCP plan. This could include taking more samples for analysis, applying additional microbiological tests, and/or applying more strict limits for acceptance. Such rigorous testing would only be done at infrequent intervals and could apply to end-product testing, environmental testing, or microbiological testing at a CCP that would normally be monitored by nonmicrobiological means. The second strategy is to apply trend analysis to the additional microbiological testing and to take more frequent samples for analysis but to use a low sampling rate. Such data could then be subject to standard statistical analysis using control charts such as Shewart or Cusum charts (see Chapters 58 and 60). However, the confidence of decisions on acceptability based on the cumulative data from successive batches of product sampled at a low rate over a period of time remains poor. Thus, it has been estimated that if there is a contamination rate in a food lot of 0.1% (for instance 1 in 1,000 product units are contaminated with salmonella) and if a sampling plan of $n = 5$; $c = 0$; $m = 0$ is applied to one lot, and if the sampling plan is then repeated on an additional 9 and 29 lots, the probability of acceptance (salmonella not detected) is respectively 99.5%, 95.1%, and 86% (Dr. P. Teufel, personal communication 1996). To obtain 95% probability of detecting a contamination level of 0.1% in a food lot, and assuming a Poisson distribution of microorganisms in the food lot, 3,000 production units would need to be examined. Thus verification of HACCP applying microbiological criteria has only limited value[16] unless very large numbers of sample units are examined.

Summary. The potential uses of microbiological criteria by industry are much broader than those of a regulatory authority (Table 65–5). There are significant advantages to industry of establishing their own criteria to meet internal needs and any required in regulations. The meeting of all such requirements usually would be achieved by the application of GMP and HACCP. The use of microbiological criteria for raw material or end-product testing should not be a routine activity unless part of an HACCP-based control program. Microbiological testing may be used more frequently when considering the use of a new supplier or raw material source, introducing a new process or where there is evidence of microbiological contamination of a process or product.

65.4 CONCLUDING REMARKS

For the most part, microbiological criteria have been, and still are, based mainly on technical achievement and not on an objective assessment of need based on utility and a scientifically based assessment of risk. As noted in the introduction to this chapter, the ICMSF[35] developed an approach to establishing microbiological criteria that was based on a form of risk and hazard ranking to establish suitable sampling plans. These plans, despite some limitations inherent in their use, have stood the test of time and deserve the worldwide acceptance that they have achieved. However, since their introduction in the early 1970s, the systems of control of the microbiological safety and quality of food have evolved and the role of microbiological criteria has needed to change from a primary control role to a role essentially supporting the establishment and application of HACCP.

Microbiological criteria should define the levels of specific microbiological hazards that are considered acceptable in a foodstuff for consumer protection. If to be established for the reasons described in this chapter, they should be based on the principles adopted by the Codex Alimentarius Commission.[18] They should communicate to both industry and the regulatory authorities the level of control that must be exercised to ensure the safety and acceptability of a foodstuff. Thus, the establishment of a microbiological criterion has a valuable function. However, as we have noted and discussed in this chapter, their application as a primary means of ensuring that a food is safe to eat or that a production process was properly applied is limited, and conclusions based on their use may be misleading and may only provide a false sense of security to their user. The overriding consideration of whether or not to use microbiological testing as part of a control process should be based on whether or not it will provide necessary additional assurance of food quality or safety.

The use of microbiological risk assessment to understand the significance of microbiological hazards in relation to their probability of causing foodborne illness should enable policy makers concerned with public health aspects of food to better establish measures (including microbiological criteria) and to appropriately assign resources to effect their control. Although in its infancy as a means of establishing the risks associated with microbiological contamination of foodstuffs, microbiological risk assessment is an important first step on the path leading to the establishment of effective international and national food control policies. To this end, the ICMSF has proposed to the Codex Alimentarius Food Hygiene Committee a series of six steps for managing the microbiological hazards associated with foods in international trade based on the documents developed by the Codex Alimentarius Commission. These steps are shown in Exhibit 65–2.

It would be exceptional for industry to undertake a formal microbiological risk analysis for the reasons outlined in the first part of this chapter. Rather industry should encourage the regulatory authorities to carry out in-depth risk assessments and for industry to work with the authorities to identify appropriate risk management options, including Food Safety Objectives.[37] Such options can then be used by industry to develop specific control measures for operations throughout the food chain based firmly on the application of HACCP principles.

Exhibit 65–2 Logic Sequence for the Future Management of Microbiological Hazards for Foods in International Trade

STEP 1: Conduct a risk assessment
Apply the "Principles and Guidelines for the Conduct of Microbiological Risk Assessment" proposed by the Codex Food Hygiene Committee[24]

- The risk assessment provides the scientific basis for risk management decisions.

STEP 2: Conduct a risk management option assessment
Apply the "General Principles of Food Safety Management."[22]

- Consider establishment of an appropriate food safety standard (ie, a food safety objective).

STEP 3: Establish a food safety objective
Food safety objective. This is a statement of the maximum level of a microbiological hazard considered acceptable for consumer protection.

- A food safety objective should be technically feasible.
- The statement should include quantitative value.
- A food safety objective should be developed by a governmental body with a view to reaching consensus for a food in international trade.

STEP 4: The food safety objective should be achieved through the application of:
"The General Principles of Food Hygiene" or product specific codes and "The Hazard Analysis and Critical Control Point (HACCP) System and Guidelines for Its Application"[25]

- It should be confirmed that the food safety objective is achievable.
- The HACCP requirements must be developed separately by the food industry.

STEP 5: Establish microbiological criteria, when appropriate

- Apply "The Principles for the Establishment and Application of Microbiological Criteria for Foods"[25]
- This step should be performed by an expert group of food microbiologists, including experts with experience in the food industry.

STEP 6: Establish acceptance procedures for the food at port of entry

- Apply "Guidelines for the Design, Operation, Assessment and Accreditation of Food Import and Export Inspection and Certification Systems" (Alinorm 97/30A Appendix II)
- Acceptance, for example, could be based on a list of approved suppliers determined by inspection of facilities and operations, certification etc; microbiological testing (if criteria have been established) and/or other testing, eg pH, a_w, or container integrity.

Source: Data from ICMSF Proposal to Codex Alimentarius Food Hygiene Committee 1997/1998.

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